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TABLE OF CONTENTS

	PAGE
Some Conditions Affecting the Growth and Activities of Azotobacter chroococcum ..... E. R. Allen	1– 44
An All-Glass Nitrogen Apparatus .. E. R. Allen and B. S. Davisson	45– 48
Arcangeliella, Gymnomyces, and Macowanites in North America .. S. M. Zeller and C. W. Dodge	49– 59
The Use of the Colorimeter in the Indicator Method of H Ion Determination with Biological Fluids... B. M. Duggar and C. W. Dodge	61– 70
Tyrosin in the Fungi: Chemistry and Methods of Studying the Tyrosinase Reaction ..... C. W. Dodge	71– 92
Studies in the Physiology of the Fungi. VI. The Relation of Bacteria to Cellulose Fermentation Induced by Fungi, with Special Reference to the Decay of Wood. Henry Schmitz	93–136
Studies in the Physiology of the Fungi. VII. Growth of Wood-Destroying Fungi on Liquid Media S. M. Zeller, Henry Schmitz and B. M. Duggar	137–142
Merulius in North America, Supplementary Notes ..... E. A. Burt	143–145
Seed Disinfection for Pure Culture Work ..... H. C. Young	147–158
Seed Disinfection for Pure Culture Work: The Use of Hypochlorites B. M. Duggar and Anne W. Davis	159–170

PAGE

An Edible Garden Hebeloma . . . . . E. A. Burt 171–174

Protomerulius Farlowii Burt, n. sp . . . . . E. A. Burt 175–177

The Micro-Colorimeter in the Indicator Method of Hydrogen Ion Determination . . . . . B. M. Duggar 179–181

Studies in the Physiology of the Fungi. VIII. Mixed Cultures . .
S. M. Zeller and Henry Schmitz 183–192

Studies in the Physiology of the Fungi. IX. Enzyme Action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr. . . . . .
Henry Schmitz and S. M. Zeller 193–200

Studies in the Physiology of the Fungi. X. Germination of the Spores of Certain Fungi in Relation to Hydrogen Ion Concentration . . . . . R. W. Webb 201–222

Diastase Activity in Relation to Stage of Development and Carbohydrate Content of the Tuber of Solanum tuberosum . . . . . R. A. McGinty 223–251

The Thelephoraceae of North America. XI . . . . . E. A. Burt 253–280

A Subterranean Algal Flora . . . . . .
G. T. Moore and Joanne L. Karrer 281–307

Culture Experiments with Melampsora in Japan . . . . . Takashi Matsumoto 309–316

General Index to Volume VI 317–320

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SOME CONDITIONS AFFECTING THE GROWTH AND ACTIVITIES OF AZOTOBACTER CHROOCOCCUM

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INTRODUCTION

The problem of soil biology is concerned to a considerable extent with studies of the activities of the oligocarbophilous and of the oligonitrophilous bacteria. Representatives of both the groups appear to be very widely distributed, and the inference is that they are more or less active in all normal arable soils. Of the former group the *Nitrosomonas, Nitrosococcus,* and the *Nitrobacter* of Winogradsky ("the nitrifying bacteria") are the most widely known, while of the latter group the *Azotobacter* and the *Bacillus radicicola* or legume bacteria ("the nitrogen-fixing bacteria") are the most familiar examples. All of these and related forms have been the subjects of extended research, and consequently an immense and growing literature exists on this general subject.

Although the physiological powers and the presence in ordinary soil of these organisms can be readily proven by suitable incubation experiments with soils or impure culture, attempts to isolate and grow these organisms, especially the *Azotobacter* and the nitrifiers, in synthetic media of entirely known composition lead to very great difficulties. Pure cultures are not readily isolated, and when obtained their growth on media of entirely known composition is so slow that inves-

tigations of the mechanism of their activities have not been particularly inviting problems to most workers. This condition or set of conditions has resulted in the production of a large amount of "soil" and crude culture work and a comparatively small amount of true physiological work. Many students of these problems have contended that this is as it should be; that pure cultures in completely synthetic media are so unnatural that results can have but little practical bearing. It seems to us that this type of reasoning is unsound and that it can never be productive of thoroughly reliable either practical or purely scientific work. This point has been discussed at length by Allen and Bonazzi ('15) with especial reference to the study of nitrification. Existing methods of work were criticized, and the difficulties to be encountered in the improvement of methods discussed. Since then, in line with this method of attack, has appeared the work of Allen ('15) and of Davisson and his co-workers ('16, '18, '19) on improvement of methods of nitrogen determinations, and of Bonazzi ('19, '19[a]) on the nitrifying bacteria. The work reported in this paper deals with experiments on *Azotobacter chroococcum,* and they have proved to be as crude and erratic as were those reported earlier on nitrification, yet just as illustrative of the difficulty of the problem and just as suggestive, we hope, of possible methods of attack.

HISTORICAL

To review in detail all the difficulties that have been reported in studies of *Azotobacter* since the organism was discovered by Beijerinck in 1901 is wholly unnecessary at this time. From numerous and diverse sources it is evident that ordinary synthetic culture media are lacking in something for pure culture work, and that aqueous soil extract or even tap water is superior to distilled water, but that the addition of a small amount of soil to the culture medium is far better.

A step forward was made by Krzemieniewski ('08), who found that humus was the important constituent of the soil for *Azotobacter,* and that the activating substance in the soil

and more particularly in the soil humus was difficultly soluble in water. The work of Krzemieniewski has rightly been the subject of much discussion, and no wholly satisfactory explanation has been brought forward yet to explain the remarkable results obtained on the addition of humus to Beijerinck's mannite culture solution. The explanations that have been suggested are, however, well worthy of note.

Kaserer ('10) conceived the idea of humus supplying certain rare or unusual inorganic constituents to the culture medium which were required in very small amounts by *Azotobacter,* but entirely essential nevertheless to their proper development. After many experiments with media containing iron, aluminium, manganese, and silicon, he considered that he had very nearly duplicated in a synthetic way the remarkable results of Krzemieniewski, which success he attributed to the presence of iron and aluminium silicophosphates which furnished iron and aluminium in soluble form to the bacteria. He believed, however, that he had not yet attained the best possible combination of required mineral nutrients. Later ('11[a]) he elaborated to a considerable extent on this theory, postulating that all bacteria require these rather unusual elements and compounds to a certain extent, but that the requirements in this line of the oligocarbophilous and of the oligonitrophilous forms were especially high. The decoctions and broths on which the ordinary saprophytic and pathogenic bacteria are ordinarily grown contain sufficient of the above compounds to cover the needs of these organisms, hence their presence has remained undetected. He predicts that before the ideal culture medium is attained minute amounts of other substances will have to be introduced, e. g., copper, zinc, arsenic, iron, and titanium. He also believes that the necessity of these unusual elements for green plants cannot be denied with certainty.

Remy and Rösing ('11) made an extended study of the cause of the results obtained from humus by Krzemieniewski. After confirming certain important points in regard to the beneficial action of humus, they proceeded to search for an explanation for such action. Kaserer's solution containing

iron, aluminium, silicates, and phosphates proved valueless in their hands, but a faintly alkaline mannite solution containing iron and aluminium phosphates appeared promising. Following this out through a series of experiments they finally succeeded in preparing a nutrient medium which contained, in addition to the regular constituents of Beijerinck's nutrient, a so-called "ferric hydroxide solution," with cane sugar as a protection against precipitation ("Fallingsschutz") by alkalis. This iron-containing solution was prepared by dissolving in 1 liter of water 1 gm. of $FeCl_3$, 10 gms. cane sugar, and 0.80 gm. iron-free NaOH. Two drops of H_2SO_4 were then added. This final solution contained 0.50 gm. Fe_2O_3 and 0.26 gm. free NaOH per liter. Numerous distinctly beneficial results are reported from the addition of Fe_2O_3 to Beijerinck's nutrient solution in the above form. For instance, when 15 mgs. Fe_2O_3 were added to 100 cc. of Beijerinck's solution 7.88 mgs. of nitrogen were fixed by *Azotobacter* per gm. of mannite in 2 weeks, whereas ordinarily only 1 to 2 mgs. were assimilated per gram of mannite.

By increasing the amount of Fe_2O_3 Remy and Rösing state that better results are obtained, although they unfortunately omitted the nitrogen determinations for these larger amounts of iron. The other substances, NaCl, cane sugar, and NaOH, present in the so-called ferric hydroxide solutions, were without effect, hence these workers felt that the following conclusions were justified:

(1) A relation exists between the iron content of the nutrient solution, *Azotobacter* development, and nitrogen fixation. In the case of ferric silicate the optimum lies above 10 mgs. Fe_2O_3 per 100 cc. of Beijerinck's mannite solution.

(2) The value of the iron varies according to its form. The most favorable is an alkaline solution in which iron hydroxide is dissolved by means of cane sugar. Then follows ferric silicate, while all other iron compounds stand far below. Thomas phosphate acts strongly, which is to be attributed in part to the content in silicic acid and basic lime.

In discussing the results of their work Remy and Rösing point out that the action of the iron cannot be that of a nutri-

ent, since the optimum lies too high. They call attention to the fact that H. Fischer suggested the rôle of humus in the soil to be that of an oxygen carrier, and that Bonnema suggested that Fe_2O_3 is the real agent in nitrogen-fixing processes, since in contact with the air it transforms continually small amounts of nitrogen into nitrite, and that this continually formed nitrite is then transformed by the microörganisms into cell protein. On these points Remy and Rösing state that they would soon offer experimental evidence, but to our knowledge it has not appeared.

Kaserer ('12) believes that the results obtained by Remy and Rösing were due to the impurities in the iron compounds used rather than to the iron itself, but Rösing ('12) does not accept this explanation.

Söhngen ('13) studied to some extent the conditions for promoting the growth of *Azotobacter,* the nitrifying bacteria, and bacteria in general. As concerns *Azotobacter* he states that the results of Krzemieniewski, Kaserer, and Remy and Rösing were in general confirmed. In addition he found that colloidal silicic acid when added to Beijerinck's medium produced an even more beneficial effect than the raw humus which he used. With Beijerinck's solution alone he obtained 1.9 mgs. nitrogen fixed per gm. of mannite, whereas when 2 gms. raw humus were added to 100 cc. of culture solution 6.7 mgs. were fixed, and when 500 mgs. colloidal silicic acid in the sol form were added 8.0 mgs. of nitrogen were fixed per gram of mannite. All the results cited were obtained with a pure culture in a 32-day incubation period.

Söhngen also obtained excellent results by inserting a strip of filter paper or of cotton cloth in the culture medium. *Azotobacter* grew almost exclusively in contact with the filter paper or cloth at or just above the junction of the strips with the nutrient solution. This indicates, according to him, that microbial life in the soil takes place chiefly upon the colloids. He believes that the beneficial action of the colloidal silica and of the raw humus is due to the fact that these colloids adsorb nitrogen and oxygen, and in this manner impart more quickly the necessary elements, and better growth results.

In further confirmation of his theory Söhngen measures the oxygen and nitrogen adsorbed by colloidal silica and colloidal ferric oxide. He finally concludes that for luxuriant development of *Azotobacter* in Beijerinck's medium only nitrogen and oxygen are lacking. By the use of the colloids described by him there occurs a direct contact between the bacteria and oxygen and nitrogen, with the result that luxuriant growth of *Azotobacter* takes place in the culture medium.

Still another theory has been proposed by Bottomley ('14) to account for growth stimulation in cultures of *Azotobacter* and nitrifying bacteria. Marked acceleration of growth of these bacteria and of wheat seedlings was obtained by him by the use of extracts of "bacterized" peat, i. e., peat which had been inoculated with certain aërobic bacteria and incubated for a suitable time under favorable conditions as to temperature and moisture. He later ('15) proposed the term "auximones" for these accessory substances, which he believed were analogous to the "vitamines" of animal physiology. Bottomley's associate, Miss Mockeridge ('17), studied in some detail the action of these extracts of bacterized peat, and believed that "auximones" had been responsible for the marked results obtained by Krzemieniewski on the addition of humus to cultures of *Azotobacter*. Bottomley ('17) claims to have isolated a nucleic acid derivative from "bacterized" peat and suggests that it is of importance in the results obtained on accelerated growth.

Very recently Bonazzi ('19) has obtained results on the growth of nitrifying bacteria that are very much to the point on this general problem. Intensive growth as measured by one physiological activity was obtained in case of *Nitrosococcus* (the nitrite-producing bacteria) when the solution was properly stirred mechanically. The nutrient medium was of very simple composition, so that the possibility of "auximones" and "rare elements" would be eliminated. It was also soon observed that this type of treatment was distinctly beneficial for the growth of *Azotobacter*. Aside from mentioning that the shaking possibly produced better aëration or assisted in the removal of by-products, Bonazzi did not enter into any

speculations as to the cause of the benefit derived from the mechanical stirring.

None of the theories which have been mentioned above will fit all the facts in regard to hastening the growth of *Azotobacter* or of the nitrifying bacteria. These theories have, however, been most suggestive and helpful in furnishing a stimulus to the investigation in this difficult field. It seems to us that the only way to proceed with the formulation of a theory is to keep trying, and to test the theories as they are proposed from as many viewpoints as possible. In this way only is it possible, it seems to us, to avoid the performance of an almost endless amount of empirical work.

Now, in viewing the results obtained from diverse sources on the stimulated growth of *Azotobacter* and of the nitrifiers, we find that in all cases growth is very slow in pure cultures in solutions under normal conditions of completely known composition. There appears to be universal agreement on this point. No one doubts, apparently, on the other hand, that the addition of humus to cultures of *Azotobacter* is quite effective, and that vigorous growth of the nitrifiers may be quite easily produced in all normal soils.

In trying to find a common factor to account for all the various improvements in culture solutions it should be borne in mind that Kaserer obtained his beneficial results from a "silicophosphate," and that Bottomley believes his "auximones" to be a nucleic acid derivative, therefore a carrier of phosphorus. We must bear in mind also that Gerlach and Vogel ('03), after studying the mineral requirements of *Azotobacter chroococcum,* concluded that phosphorus and calcium were absolutely indispensable nutrients. After a 67-day incubation period they found the following nitrogen relations per 1,000 cc. of nutrient solution:

		Flask content (mgs. N)	Gain over control (mgs. N)
Series	I (all inorganic nutrients)	45.2	42.5
Series	II (without calcium).....	3.1	0.4
Series	III (without potassium).....	21.6	18.9
Series	IV (without phosphorus).....	2.8	0.1

	Flask content (mgs. N)	Gain over control (mgs. N)
Series V (without potassium and phosphorus)	2.9	0.2
Series VI (without sodium)	18.0	15.3
Series VII (without potassium and sodium)	21.2	19.5
Series VIII (all nutrients, uninoculated)	2.7	

In addition to these essential elements it is quite evident—although absolutely definite data are lacking—that the reaction of the medium is quite important. It is known beyond any question that the medium must not be acid, but the exact concentration of hydrogen as ion has not been properly studied. It has been more or less generally considered that the reaction should be faintly alkaline, and the fact that the addition of solid calcium carbonate to the culture medium, which is then maintained or "buffered" to a reaction of approximately P_H 8.0, has found quite wide favor, is in accord with this idea.

Now it is evident at once that these three requirements, phosphates, calcium, and a faintly alkaline solution, are very difficult to obtain, owing to the formation of the but slightly soluble tricalcium phosphate. For instance, in Ashby's medium, which is prepared from distilled water, mannite (or dextrose), potassium phosphate, sodium chloride, calcium and magnesium sulphates, a trace of ferric chloride and solid calcium carbonate, the phosphates are essentially quantitatively transformed into the almost insoluble tricalcium phosphate. In Beijerinck's medium, which is made from tap water, mannite (or dextrose), and dipotassium phosphate, the calcium (contained in the tap water) is precipitated during the sterilization processes owing to the hydrolysis of the dipotassium phosphate.[1] Thus Ashby's solution is deficient in soluble phosphates, but is well buffered slightly on the alkaline side, while Beijerinck's solution is lacking in soluble calcium salts, its only buffer being a relatively low amount of dipotassium phosphate, which maintains its reaction approximately at neu-

[1] In Winogradsky's medium for nitrifying bacteria the phosphorus is precipitated as ferric, ferrous, and magnesium phosphates.

trality at incubation temperatures.[1] The addition of calcium carbonate, therefore, to Beijerinck's solution introduces two variables: It changes its reaction and it precipitates the phosphates. It is not surprising, therefore, that the tendency has been to omit calcium carbonate in Beijerinck's solution, especially when mannite is used as the energy source.[2] In Ashby's solution more soluble calcium (as sulphate) is added than in Beijerinck's solution, hence more of the phosphate is carried down, with the result that the solution is very poorly buffered and hence unsuited to the growth of *Azotobacter* (especially when dextrose is used), unless calcium carbonate be supplied to each culture vessel. Although direct comparisons are few or wanting entirely, it seems from the literature that Beijerinck's solution is preferable to Ashby's. In the light of the above reasoning it may easily be that this is because it contains more soluble phosphate than does Ashby's and that this, besides being more available as a nutrient, acts as a soluble buffer and as such is more effective than the solid calcium carbonate.

Now it was conceivable to us that the phosphorus nutrition and possibly the maintenance of proper hydrogen ion concentration separately or in conjunction were operative in all the above-cited cases of growth stimulation of *Azotobacter chroococcum*. For instance, in Krzemieniewski's experiments the organic combinations of calcium may have supplied this element in an assimilable form and organic phosphates in the humus may also have been beneficial. The colloids which Söhngen used might have acted as protective colloids, i.e., prevented the complete flocking out of the tricalcium phosphate after it was once formed. The properties of soil grains which he believed was due to adsorption of gases may have been due to their adsorbed phosphate ions, or possibly to the difficultly soluble phosphate compounds precipitated as a thin film as a

[1] The exact P_H value is influenced by the mannite or the dextrose present.

[2] When dextrose is added it, of course, shifts the reaction slightly toward the acid side, because of its properties as a weak acid. (See Mathews, A. P. Physiological Chemistry, p. 32. 1916.)

part of the coatings of the grains.[1] So far as culture solutions are concerned, however, it seems to us that the action of the colloid as a protection against precipitation of tricalcium phosphate is a simpler explanation.

In considering the work of Kaserer and of Remy and Rösing, particularly of the latter, the fact must not be lost sight of that the terminology used by them with respect to solutions is very loose in the light of modern chemistry. The "ferric hydroxide solution" of Remy and Rösing is, of course, a colloidal suspension of hydrated ferric oxide, and as such it might easily possess the property of a protective colloid and prevent the flocking out of phosphates in the culture medium. Kaserer's postulation of a silicophosphate as a chemical compound is unwarranted. It is much more likely that his "iron aluminium silicophosphate" is a colloidal suspension of varying composition and stability carrying with it some tricalcium phosphate in colloidal suspension.

As concerns Bottomley's results, it has already been mentioned that he considers the benefit observed by him to be due to compounds containing phosphorus in the organic form. Regarding the results obtained by Bonazzi with mechanical agitation, it is clear that "auximones" and "rare elements" are eliminated, although "oxygen carriers" and the "adsorption of gases" might really have the same action as that of the shaker. It seemed more probable to us that the precipitate which formed in the medium contained phosphates absolutely essential to the development of the microörganisms, and that the agitation hastened the restoration of the concentration of the solution in the equilibrium which exists between this solid phase and the nutrient solution as the materials are

[1] Söhngen considers the possibility of adsorption phenomena being operative. For instance, he says: 'The equilibrium between the concentration of the dissolved substances in the soil water and the nonorganized colloids is therefore continually disturbed in consequence of the metabolism of the organized colloids. Yet the concentration of substances in the soil water is maintained more nearly constant than in a medium without colloids, for the reason that the nonorganized colloids give up again the compounds obtained from the soil water because of the new state of equilibrium between the concentration in the liquid and the colloid. . . . . There is therefore a continual exchange of assimilable compounds between the organized and the nonorganized colloids, by virtue of which the mass of the nonorganized colloids serves as a storehouse with assimilable compounds, which is daily filled, and out of which the organized colloids regularly feed.'

assimilated, i. e., removed from solution, by the growing forms.

Thus it seems that phosphorus nutrition is associated more or less intimately with the growth and development of the *Azotobacter*. The problem then to us seems to be in part a question of supplying phosphates in the presence of calcium in a slightly alkaline solution. To test our hypothesis the set of experiments described below has been carried out. The theory is naturally quite difficult to prove or disprove, especially in view of the experimental difficulties encountered in work of this kind, and while our results have not led to positive conclusions one way or another it seemed that in view of the uncertainty of carrying work to completion at the present time it was best to report what findings we have, with the hope that they may be of value as suggestions to others as well as to ourselves.

Experimental

Culture used.—The *Azotobacter* used was a subculture from a strain of *Azotobacter chroococcum* isolated by Mr. A. Bonazzi from Wooster, Ohio, soil. It was repeatedly plated during the isolation until it gave a uniform microscopic picture and produced on Ashby's mannite agar plates circular colonies, edges entire, moderately raised and shiny. Its purity was assured during this work by repeated platings on mannite agar similar to Ashby's in composition. The colonies on agar plates appear, in 24 hours, small, round, and translucent. They grow rather slowly and become opaque, grayish white, and up to 4 or 5 days are of almost butyrous consistency, while as the culture ages they gradually become dry and at 10 days present a slightly wrinkled condition, while at 12 to 14 days the growth is dry, distinctly wrinkled, and shows a faint production of black pigment. The growth of *Azotobacter* on agar slant is entirely similar to the above, although, of course, in consequence of its slimy consistency it shows a fairly marked invasion at the base of the slant. Grown on modified Ashby's soil extract (1:2) mannite agar, it soon acquires a marked pigmenting power, which it loses again when grown on purely

synthetic media. In culture solutions it manifests itself by the formation of a translucent zoögloea mass at the junction of the solution surface and the walls of the container. At from 4 to 6 days this largely disappears and a fine white sediment appears at the base of the flask. In no case did we observe even the suggestion of the surface scum or pellicle which is widely described in the literature.

The purity of the culture also was checked by numerous microscopic examinations. The regular method for making microscopic preparations was staining for 5 minutes with dilute 1:10 aqueous methylene blue, clearing in xylol-alcohol, equal parts, and then in xylol. Preparations were also made, using ordinary carbol gentian violet and clearing as above. The cells from very young cultures (12 hours) show little differentiation in structure when stained with ordinary carbol gentian violet or with dilute aqueous methylene blue, and tend to be oval or bacillary in form. Slightly older cultures (e. g., 60 hours) show some differentiation and the appearance of granules is more frequent. Cells from cultures 4 to 6 days old appear as large, thin-walled, decidedly granular cocci or diplococci, while preparations from old cultures (e. g., 15 days) generally show thick-walled cocci surrounded by considerable slime, the cell contents failing to take either of the above stains appreciably (pl. 1, figs. 1–6). The nature of these cytological differentiations has been discussed by Bonazzi ('15).

The culture when obtained was in its twenty-fifth transfer from soil, and transfers and platings made from this time on were designated as F_1, F_2, F_3, etc.

Culture medium.—The standard culture medium used in this work was essentially a modified Ashby's medium of the following composition:

Mannite	20	gms.
Monopotassium phosphate	0.2	gm.
Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Calcium sulphate	0.1	gm.
Distilled water	1000	gms.
10% ferric chloride solution	2	drops

Agar was prepared by the addition of 1.5 per cent Bacto[1] agar to the above. Soil extract agar was prepared by substitution of 1:2 soil extract in place of distilled water. A pinch of $CaCO_3$ was added to each agar tube or culture vessel. In platings special care was taken to get the $CaCO_3$ well into suspension before pouring, as growths on the plates were better when this precaution was taken.

Although, as stated above, the Beijerinck medium is probably superior to Ashby's, we chose the latter, as it was of known composition, whereas the use of tap water introduced unknown factors. Beijerinck's solution was used therefore only in a few cases for purposes of comparison.

Our experimental work is best considered under three separate heads: (1) the preliminary or orientation work which is concerned chiefly with the checking of important results; (2) the improvement of experimental methods; and (3) the final experiments which are designed to throw light on the reason for improved growth in cultures of *Azotobacter* mechanically agitated.

ORIENTATION EXPERIMENTS

It seemed to us that it was well worth while to attempt to duplicate certain phases of Remy and Rösing's work. At the outset we experienced some difficulty in preparing a stable colloidal ferric oxide solution according to the directions of Remy and Rösing. This we attributed to the presence of sulphate ion. After a few empirical experiments, a colloidal ferric oxide solution was prepared as follows: One gm. Kahlbaum's pure ("zur Analyse") $FeCl_3$ and 10 gms. of saccharose were dissolved in approximately 700 cc. of distilled water, 0.34 gm. Na_2CO_3 added, the solution heated till perfectly clear, cooled, and made to 1 liter. This solution was then strongly reddish in color, and showed no tendency to precipitate when heated to boiling, although a small portion of it did precipitate when added to Ashby's solution and autoclaved. Assuming that the $FeCl_3$. 6 H_2O was pure, the solution contained .295 gm. Fe_2O_3 per liter. This iron-sugar solu-

[1] Prepared by Digestive Ferments Co., Detroit, Mich.

tion was carefully checked in regard to its nitrate content. A nitrate determination on a 5-cc. portion by the modified Devarda method did not give an amount of nitrogen detectable with N/50 acid. A blank solution was prepared containing saccharose and NaCl equivalent to the $FeCl_3$ and added to the different cultures in the amounts indicated below.

Two series of cultures were prepared, the one employing 100 cc. of Ashby's solution in 300-cc. Erlenmeyer flasks, the other the same amount in 700-cc. Erlenmeyers. The culture solution was prepared as indicated above, double distilled water being used. The reaction was carefully adjusted to the phenolphthalein neutral point and approximately ½-gm. portions of c. p. $CaCO_3$ added to each flask. One-mg. and $\frac{1}{10}$-mg. portions of colloidal Fe_2O_3 were added to certain of the culture solutions. These amounts were supplied by the addition of the proper amounts of the iron-sugar solution or of dilutions prepared from it. Corresponding amounts of the NaCl sugar solutions were added to the controls.

The culture solutions were inoculated with a suspension prepared from a 72-hour slant of F_4 on Ashby's soil extract agar. As much of the growth as could be removed with a spiral was transferred to a 10-cc. water blank, well shaken, and one spiral of the suspension used for inoculating each flask of culture medium. The cultures were incubated for two weeks in a warm room, after which they were analyzed for total nitrogen by the Kjeldahl-Gunning method. The contents of the cultures were transferred to 500-cc. Kjeldahl flasks with ammonia-free water, the complete transfer of the culture material being assisted by the addition of the 30 cc. of concentrated sulphuric acid in three 10-cc. charges to the Erlenmeyer flasks and subsequent washings into the Kjeldahls. Ten gms. of anhydrous sodium sulphate and 2 cc. of 10 per cent copper sulphate solution were added and digestion carried out in the regular manner. Boiling was continued for 1½ hours after the solutions became clear. After cooling the melt was treated with 200 cc. of nitrogen-free water, 60 cc. of 50 per cent alkali then added, and distillation performed with an apparatus essentially the same as that used previously

for nitrate determinations (Allen, '15, fig. 1), N/50 acid being used in the receivers. Bumping was prevented by the addition of zinc.

Extreme difficulty was experienced in carrying out the digestions. Foaming was excessive and the cultures reported "lost" in table I were those which foamed over. Only a very low flame could be used and the rate of digestion was extremely slow. The large amount of carbonaceous material, which formed from the decomposition of the mannite, was extremely resistant to digestion, and it was only after several days of slow intermittent boiling that the mixtures cleared. After the determinations on these preliminary experiments were completed, some studies on optimum conditions for digestion were taken up. These are discussed separately.

The results of the first of the preliminary experiments are given in table I. The control cultures received, as stated above, sodium chloride and cane sugar equivalent to the ferric oxide and cane sugar in the remaining flasks.

TABLE I

EFFECT OF COLLOIDAL FERRIC OXIDE IN FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN DEEP AND SHALLOW LAYERS

No.	Additions to Ashby's culture medium	Treatment	300-cc. flasks		700-cc. flasks	
			N found (mgs.)	N fixed (mgs.)	N found (mgs.)	N fixed (mgs.)
1	Control	Sterile	Lost	.....	1.31	.....
2	Control	Inoc.	3.23	1.56*	2.59	1.28
3	Control	Inoc.	2.84	1.17	1.98	0.67
4	0.1 mg. Fe_2O_3	Sterile	1.67	.....	1.34	.....
5	0.1 mg. Fe_2O_3	Inoc.	5.08	3.41	7.94	6.60
6	0.1 mg. Fe_2O_3	Inoc.	4.52	2.85	Lost	.....
7	Control	Sterile	1.34	.....	1.45	.....
8	Control	Inoc.	2.33	0.99	2.60	1.15
9	Control	Inoc.	1.80	0.46	3.99	2.54
10	1.0 mg. Fe_2O_3	Sterile	Lost	.....	1.22	.....
11	1.0 mg. Fe_2O_3	Inoc.	2.72	1.38†	Lost	.....
12	1.0 mg. Fe_2O_3	Inoc.	Lost	.....	4.73	3.51

* Computed from No. 4 as blank. † Computed from No. 7 as blank.

While the data are erratic and incomplete, they indicate that the 0.1-mg. portion colloidal ferric oxide exerted a beneficial effect on the growth of the microörganism. The growth

in the deeper layers is unsatisfactory, as would be expected from the work of others.

The effect of colloidal ferric oxide in Beijerinck's[1] solution was also studied. The nutrient solution contained 20 gms. mannite and 0.2 gm. KH_2PO_4 per 1000 cc. of tap water. A portion was titrated with phenolphthalein, and the calculated amount of normal NaOH added to make the medium very faintly alkaline to phenolphthalein. For the sake of determining the effect of tap water, a second medium was prepared in exactly the same manner except that redistilled water was used. One-hundred-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyers, and then 1.0 mg. of colloidal Fe_2O_3 in the form of the solution described above was added to each flask. Since the concentration of salts is less in the Beijerinck solution than it is in the Ashby, and since it lacks $CaCO_3$ besides, it was reasonable to expect that the colloidal Fe_2O_3 would remain in suspension better in it than in the Ashby. While no difference could be detected in the unheated solutions, after autoclaving the Beijerinck medium appeared to be more colored than the Ashby, although partial precipitation occurred in both. The data obtained after a two weeks' incubation at 28–32° C. are reported in table II below. For the sake of comparison the data on Ashby's solution containing 1.0 mg. Fe_2O_3 per culture of 100 cc. are retabulated.

TABLE II

GROWTH IN PRESENCE OF 1.0 MG. OF COLLOIDAL FE_2O_3 IN DIFFERENT NUTRIENT SOLUTIONS

No.	Medium	Treatment	Water	N found (mgs.)	N fixed (mgs.)
10	Ashby	Sterile	Redistilled	1.22	.....
11	Ashby	Inoc.	Redistilled	Lost	.....
12	Ashby	Inoc.	Redistilled	4.73	3.51
19	Beijerinck	Sterile	Redistilled	1.05	.....
20	Beijerinck	Inoc.	Redistilled	3.35	2.30
21	Beijerinck	Inoc.	Redistilled	2.88	1.83
22	Beijerinck	Sterile	Tap	1.13	.....
23	Beijerinck	Inoc.	Tap	Lost	.....
24	Beijerinck	Inoc.	Tap	7.70	6.57

[1] In the original Beijerinck medium dipotassium phosphate is used, and, of course, no neutralization to phenolphthalein is necessary.

It seems that the Beijerinck solution prepared with tap water (i. e., the true Beijerinck solution) is superior to Ashby's solution when equal amounts of colloidal Fe_2O_3 are added to each. The loss of culture 23 is very unfortunate, but the notes describe cultures 23 and 24 as rapid and vigorous growths, as indicated first by turbidity, then zoögloea formation at junction of liquid surface with walls of glass flask, and later by the formation of a finely flocculent precipitate at the bottom of the flask.

The growth in cultures 20 and 21, while far below that in the regular Beijerinck solution, is really greater than we had reason to expect. This indicates that needs of the organism for mineral nutrients, aside from sodium, potassium, phosphorus, and iron, must be very low indeed and were partly covered by the impurities carried by the constituents of the medium.

It is interesting to note in this connection that it is more or less generally recognized by bacteriologists that tap water is superior to distilled water for the preparation of regular media. The objection to its universal use is its inconstancy of composition and the consequent varying results which attend its use in different laboratories or even in the same laboratory at different times. Now, soil biologists recognize the significance of this factor and moreover that in many cases soil extract is superior to tap water. In the case of *Azotobacter,* for instance, it is quite generally known that Ashby's soil extract medium is superior to the regular Ashby medium in which distilled water is used, and to that end we used Ashby's soil extract agar to some extent in the propagation of our stock culture. It is also universally recognized by chemists, and to a less extent by biologists, that distilled water is not free from dissolved substances. Inorganic salts are carried over mechanically entrained in the vapor, and volatile organic compounds are with difficulty completely destroyed. It seemed worth while in this work to remeasure the magnitude of the differences resulting from the use of tap, distilled, and redistilled water. The stock laboratory distilled water was prepared by an electric still with a preheating device, and was

stored in a tin-lined copper tank and from here distributed to the laboratories by a system of block tin tubes. The redistilled water was prepared by distilling this water over acid permanganate. The connection between the block tin condenser and the distilling flask was made by wadding with absorbent cotton. The results obtained are reported in table III below. The data on redistilled water are, it will be noted, those reported before in table I as cultures 1, 2, and 3.

TABLE III

INFLUENCE OF DIFFERENT WATERS USED IN PREPARATION OF MODIFIED ASHBY'S NUTRIENT SOLUTION

No.	Water used	Treatment	N found (mgs.)	N fixed (mgs.)
1	Double distilled	Sterile	1.31	.....
2	Double distilled	Inoc.	2.59	1.28
3	Double distilled	Inoc.	1.98	0.67
13	Stock distilled	Sterile	1.03	.....
14	Stock distilled	Inoc.	3.58	2.55
15	Stock distilled	Inoc.	2.78	1.75
16	Tap	Sterile	1.26	.....
17	Tap	Inoc.	3.49	2.23
18	Tap	Inoc.	4.45	3.19

The differences observed are not wide but serve to show the order of magnitude of the effect produced by different waters, and also illustrate the fact that something is lacking in Ashby's solution.

As mentioned above, very great difficulty was experienced in digesting the cultures according to the Kjeldahl-Gunning method. We decided next to see to what extent this difficulty could be overcome by using a very much smaller amount of material, a principle that finds extended use in the "micro" methods of biological chemistry. Accordingly, 10-cc. portions of Ashby's solution were placed in 60-cc. Erlenmeyer flasks. The medium was made as above except that tap water was used. Calcium carbonate was omitted in one-half the culture flasks, since, as mentioned above, it seemed to exert a quite marked precipitating effect on the colloidal ferric oxide which was added in the amounts of 0.01, 0.05, and .1 mg. per 10 cc. of culture solution. The flasks were inoculated with a spiral

of a suspension prepared as described above from a 24-hour-old streak of the F_2 generation on Ashby's soil extract agar. The "micro" cultures were kept in an incubator at 30–31° C. for two weeks and then analyzed for total nitrogen according to the procedure described above except that 15 cc. concentrated H_2SO_4, 7 gms. Na_2SO_4, and 2 cc. 10 per cent $CuSO_4$ were used for each digestion. As the amount of mannite was only one-tenth that in the previously described experiments, digestion was completed in much less time. It was, however, not to be designated as rapid or free from the annoyance of foaming. In fact, from 3 to 5 hours were required for the digestion mixtures to clear, and some cultures were lost as a result of foaming out of the digestion flasks.

The results are reported in table IV. The data on fixation are computed to mgs. per 100 cc., i. e., 10 times the amount actually observed.

TABLE IV

FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN MODIFIED ASHBY'S SOLUTION

No.	Addition to culture medium	Treatment	No $CaCO_3$		$CaCO_3$ added	
			N found per culture (mgs.)	N fixed per 100 cc. (mgs.)	N found per culture (mgs.)	N fixed per 100 cc. (mgs.)
25	Control	Sterile	.360	.....	.439	.....
26	Control	Inoc.	.544	1.84	.660	2.21
27	Control	Inoc.	.487	1.27	.660	2.21
28	0.01 mg. Fe_2O_3	Sterile	.329	.....	.453	.....
29	0.01 mg. Fe_2O_3	Inoc.	.646	3.17	1.357	9.04
30	0.01 mg. Fe_2O_3	Inoc.	.601	2.72	.850	3.97
31	Control	Sterile	.227	.....	.190	.....
32	Control	Inoc.	.351	1.24	.422	2.32
33	Control	Inoc.	Lost	.....	.448	2.58
34	0.05 mg. Fe_2O_3	Sterile	Lost	.....	.133	.....
35	0.05 mg. Fe_2O_3	Inoc.	.756	5.29*	.955	8.22
36	0.05 mg. Fe_2O_3	Inoc.	.674	4.47*	.949	8.16
37	Control	Sterile	.196	.....	.234	.....
38	Control	Inoc.	.334	1.38	Lost	.....
39	Control	Inoc.	.326	1.30	.521	2.87
40	0.10 mg. Fe_2O_3	Sterile	.210	.....	.215	.....
41	0.10 mg. Fe_2O_3	Inoc.	.734	5.24	.997	7.82
42	0.10 mg. Fe_2O_3	Inoc.	.615	4.05	.906	6.91

* Computed from No. 31 as blank.

The application of "micro" technique to the problem at hand did not seem especially promising, as the nitrogen determinations were still accompanied with considerable difficulty, and since the error of the analysis was almost as great, the final value computed to mgs. N per 100 cc. contains an appreciably greater error. This point is discussed again below.

Although the data are somewhat erratic, it seems permissible to conclude that the addition of colloidal iron to Ashby's solution produces a beneficial effect and also that calcium carbonate has a beneficial action even though the reaction of the culture medium is carefully adjusted beforehand, and even though it appears to cause a greater flocking out of the colloidal ferric oxide.

It seems that we can conclude with reasonable safety from the above admittedly crude results that in a general way the work of Remy and Rösing has been confirmed. The beneficial results obtained by the addition of colloidal ferric oxide to culture solutions are much less marked than those of Remy and Rösing, yet there seems to be no reasonable doubt that such action is well worth further study, particularly in the line of the rôle of the colloidal ferric oxide. Before such a study can be carried on advantageously it is necessary to make a decided improvement in experimental methods. This point will now be considered.

IMPROVEMENT IN METHODS

One of the first points to be considered in the improvement of experimental methods was that of a suitable method of sugar determination. The exact measurement of the energy consumption in cultures of *Azotobacter* is worthy of much more study than it has received. Many workers have made computations on the amount of mannite or carbohydrate added to the culture medium, disregarding the residual amount of energy-supplying material. The exact determination of mannite is not feasible, and the determinations that have been made of dextrose have been accomplished with the

use of crude and cumbersome methods that have not especially invited further work in this direction.

In the experiments reported above 10-cc. and 100-cc. portions of culture media were used. The former did not render the Kjeldahl digestion sufficiently easy, whereas the others were so large that with the containers available it was almost impossible to keep the culture solution shallow enough to permit proper growth. The proper line of improvement seemed to be, therefore, to improve conditions as to methods of digestion for cultures intermediate in size between the above extremes, i. e., having a volume of 25–50 cc. The methods of distilling and recovering the ammonia were not wholly satisfactory, and some studies of refinement of distillation methods were made.

Another source of error or annoyance in the above experiments was the matter of a uniform method of inoculating a series of flask cultures. Of course, inoculating directly from an agar slant with a platinum loop is open to considerable objection on the ground of lack of uniformity. Inoculations should preferably be as small as possible where quantitative chemical determinations are to be made on the culture, and to this end attempts were made to inoculate the culture flasks with either one cc. or a spiral of a suspension of 1 spiral of agar slant growth in 50 cc. of sterile water. Results were uncertain, in fact almost wholly negative. The point seemed therefore worthy of further study.

The work on improvements in methods (1) of sugar determination, (2) of nitrogen methods, and (3) of inoculation, will now be considered seriatim.

DETERMINATION OF SUGAR IN AZOTOBACTER CULTURES

For the determination of dextrose in cultures of *Azotobacter* it seemed to us to be worth while to attempt to adopt some of the more modern methods to the problem in hand. The method of Shaffer ('14) appeared most promising from the standpoint of ease of manipulation and accuracy of results, and, with only very minor modifications, it proved to be applicable to cultures of *Azotobacter*. The principle of the

method is that proteins are removed by the Michaelis-Rona colloidal iron precipitation, and the centrifuge used for clarifying the solution and recovery of the cuprous oxide, which is then determined by Bertrand's method.

In our first experiments dextrose was determined on one culture and nitrogen on its duplicate; later the procedure was modified so that sugar and nitrogen were determined on the same culture with reasonable accuracy. The procedure finally adopted follows: The culture medium is acidified with N/2 HCl and warmed till mineral salts are in solution and the proteins dispersed to an opalescent solution. After cooling the material is transferred to a 100-cc. or a 250-cc. volumetric flask, depending on the size of the culture, and made to the mark. An aliquot of this suspension is transferred to a 100-cc. volumetric flask, the volume made to approximately 75 cc., and a pinch of sodium acetate added to reduce the hydrogen ion concentration. Five cc. of Merck's colloidal iron are then added, the suspension well mixed, and approximately 0.2 gm. Na_2SO_4 added, and water added to the mark. The suspension is again well mixed, poured into a 100-cc. centrifuge tube, and centrifuged for 15 minutes. Duplicate 20-cc. portions of the clear supernatant liquid are then transferred to 50-cc. centrifuge tubes. The procedure from this point on is the same as that outlined by Shaffer.

The method of Shaffer is really a "micro" method proposed for the determination of sugar in blood, where only small samples of a tissue low in sugar can be analyzed. The conditions worked out by Shaffer cover naturally a comparatively narrow range of dextrose amounts; hence in working with cultures of *Azotobacter* which contain, in the controls at least, very large amounts of sugar, it is easily possible to draw off in aliquoting a too large amount of dextrose in solution. On the other hand, it is just as easy to remove an aliquot so small that the error of the analysis is multiplied by a too large factor in computing the amount of sugar in the portion of culture medium under examination. The latter error was made in some of our determinations and probably accounts for the results indicated below as being of questionable value.

We obtained most satisfactory results by aliquoting, so that the final amount reduced in the centrifuge tube corresponded to 2 cc. of 2 per cent dextrose nutrient solution.

Nitrogen is determined on the remainder of the culture solution and computed to the total amount of the original culture.

NITROGEN METHODS

Distillation.—The distillation apparatus used above, which had proved very satisfactory in the Wooster laboratory for distillations from weakly alkaline solutions, did not prove entirely satisfactory for distillations from the strongly alkaline solutions used in the Kjeldahl method. A very slight escape of ammonia was detected from the receiver flask by means of a second receiver flask. The introduction of a cooled condenser was therefore necessary. At the same time we made some experiments on an apparatus without rubber connections. This device is shown in fig. 1. The mouth of the Pyrex flask *A* was flared slightly and ground to fit the head *B* which was sealed on to the condenser tube *C*, the latter being provided with the water jacket *D* 13 inches in length and terminating in the perforated bulb *E*.

The difficulty attending the use of a cooled condenser is that complete transfer of the ammonia requires sufficient distillation to increase the volume in the receiver to a point where accurate titrations with N/50 solutions are interferred with. This difficulty is overcome to a considerable extent by the use of the principle employed by Benedict[1], i. e., distilling into the cooled condenser for 15 or 20 minutes, then draining the condenser and completing the distillation. Using the above apparatus and distilling slowly for 20 minutes through a cooled condenser, then draining and continuing the distillation for 20 minutes longer, quantitative transfer of the ammonia was effected and the volume of the receiver kept fairly low. As zinc was used to prevent bumping and as no provision for scrubbing was included in the apparatus, a second distillation over N/10 NaOH was necessary. The magnitude

[1] Benedict, F. G. The distillation of ammonia in the determination of nitrogen. Am. Chem. Soc., Jour. **22** : 259–263. *f. 1.* 1900.

of the error from the mechanical carrying-over of the alkali may be seen in table VI where "first" and "second" distillations are recorded. This apparatus was only moderately satisfactory. The volume of the receiver flasks varied from 130 to 150 cc., whereas a range of 100 to 115 cc. would have

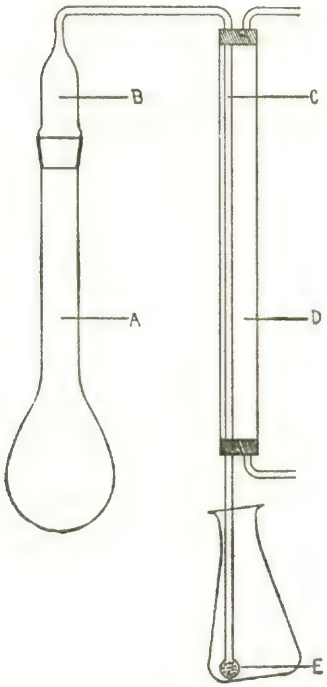

Fig. 1

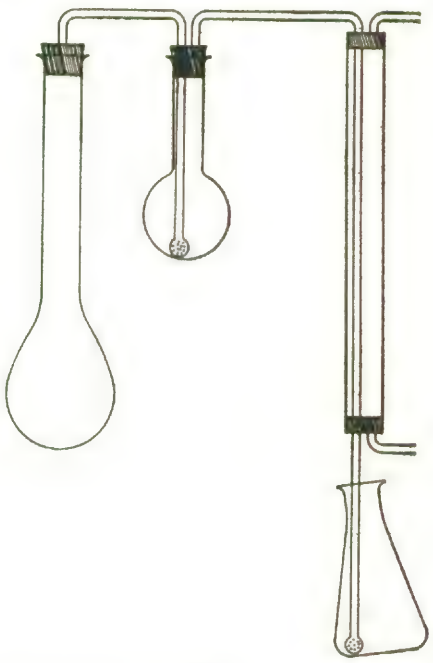

Fig. 2

Nitrogen-distilling apparatus.

been more satisfactory. Moreover, the error incident to redistillation detracts from the greater accuracy resulting from the elimination of rubber stoppers. The final apparatus adopted is that shown in fig. 2, which is self-explanatory. Pyrex glass was used throughout except for condenser jackets. Distillation was carried on slowly for 20 minutes through a cooled condenser and 20 minutes after draining the condenser. It was found by a series of distillations on ammonium sulphate solutions that quantitative recovery was effected by this procedure, and the volume of the receiver flasks as a rule remained below 115 cc. at the end of the distillation period. This

apparatus was used throughout the "Final" experiments reported below.

From this point the matter of a distillation apparatus has been developed as a separate problem and is reported in a following paper (Allen and Davisson, '19).

Kjeldahl digestions.—As stated above, the foaming accompanying solutions high in mannite was most troublesome, and it was realized that unless some improvement could be devised future progress would be almost blocked. Varying conditions with respect to catalysts, i. e., using different amounts of mercury, copper sulphate, and metallic copper, did not give any appreciable aid. The foaming appeared to be due to separation at the outset of a large amount of carbon or highly carbonaceous material which is very resistant to decomposition in boiling sulphuric acid. It occurred to us that the high temperature imparted to the digestion mixtures by the sodium (or potassium sulphate) caused a heavier deposit of this material than would otherwise be the case. Experiment proved the correctness of this suggestion, and it was found that by carrying on the digestion for 20 to 25 minutes with sulphuric acid and copper sulphate alone, then adding the sodium sulphate, the danger from foaming was slight and the digestion mixture containing 2 gms. mannite cleared in from 60 to 85 minutes.

The empirical experiments with catalysts were then repeated, with the result that copper sulphate alone seemed to be the most desirable agent. Judging by the time required for the clearing of the mixtures, mercury adds but little to the effect of $CuSO_4$ and possesses the disadvantage, of course, that Na_2S must be added to the alkali. The above experiments were made with $CuSO_4$,[1] 2.0 gms., Hg, 3 drops, and Cu, 0.5 gm. It was noted that the above amount of $CuSO_4$ was very efficient in inhibiting foaming. Hibbard[2] found that large amounts of $CuSO_4$ were associated with incomplete recovery of NH_3 by distillation. To determine the minimum amount of $CuSO_4$ required to prevent foaming varying amounts of this

[1] All references to copper sulphate refer to the hydrate $CuSO_4$ .5 H_2O.

[2] Hibbard, P. L. Notes on the determination of nitrogen by the Kjeldahl method. Jour. Ind. and Eng. Chem. 2 : 463–466. 1910.

salt were added to the digestion flasks containing 2 gms. mannite and 30 cc. of conc. H_2SO_4. After digestion had proceeded for 20 minutes, 10 gms. of Na_2SO_4 (anhydrous) were introduced into each flask. The time for the solutions to become clear bluish green and the degree of foaming were recorded. The results appear in table v.

TABLE V

EFFECT OF VARYING AMOUNTS OF COPPER SULPHATE

No.	Amount $CuSO_4$ (gms.)	Time of clearing (minutes)	Order	Foaming
1	0.1	73	3	Decided
2	0.2	68	2	Decided
3	0.3	78	4	Decided
4	0.4	80	5	Moderate
5	0.5	63	1	None

The differences in regard to time are of minor significance. The differences in regard to foaming are important and indicate that as measured by this standard less than 0.5 gm. of $CuSO_4$ should not be used. The digestion procedure used in the "Final Experiments" was as follows: The sample is digested for 20 or 25 minutes with 30 cc. of H_2SO_4 and 0.5 gm. $CuSO_4$. Ten grams anhydrous Na_2SO_4 were then added and the digestion completed. The above results were confirmed repeatedly with the slight difference that the digestion of nutrient mannite or dextrose solutions required a trifle longer than the pure mannite, this probably for the reason that the salts of the solution were slightly inhibitory in action. As a general average the mixtures cleared in 1½ hours; in all cases the digestion was continued over a low flame for 1½ hours after clearing.

METHODS OF INOCULATION

Two methods of inoculation were used, and 10 cultures seeded by each method were incubated and analyzed. By the one method, a spiral of growth from an agar slant was transferred to 10 cc. of Ashby's solution (plus $CaCO_3$) contained in a 250-cc. Erlenmeyer flask, and the culture maintained on the shaker for 24 hours in the warm room at 28–30° C. One spiral

of this suspension was then used as the inoculum. This procedure was designated as method *A*.

In the other method of inoculation a spiral of the growth on an agar slant was introduced into a regular 10-cc. water blank, well shaken, and a spiral of this used at once as the inoculum. This procedure was designated as method *B*.

Twenty 250-cc. flasks were prepared, each containing 20 cc. of Ashby's solution (plus $CaCO_3$). Ten of these were inoculated by method *A* and 10 by method *B*. For preparing the suspension in the former, the growth from a 72-hour Ashby soil extract agar slant of F_4 generation was used, while for method *B*, material from the same slant 76 hours old was used; that is, all flasks were seeded on the same date. After inoculation the cultures were incubated on the shaking machine[1] in the warm room which remained at 28–30° C. except one night when it dropped to 22° C. for several hours. Cultures inoculated according to method *A* showed a distinct and uniform turbidity on the third day, while those inoculated according to method *B* showed no visible growths until the fourth day, and these were less distinct and less uniform than those observed in the other set at 3 days. An incubation period of only 5 days was used, as it was believed that a short period

TABLE VI
COMPARISON OF METHODS OF INOCULATION

Culture no.	Inoculated according to:			
	Method A		Method B	
	1st dist. (mgs. N)	2nd dist. (mgs. N)	1st dist. (mgs. N)	2nd dist. (mgs. N)
1	2.26	1.31	2.41	0.97
2	1.59	1.26	2.44	0.93
3	2.01	1.14	1.18	1.10
4	1.64	1.10	1.97	0.92
5	6.08	1.14	Lost*	0.81
6	4.05	1.51	1.10	0.96
7	2.31	1.03	1.71	0.78
8	3.34	1.12	1.11	0.97
9	3.07	1.21	1.81	0.83
10	1.74	1.03	1.67	1.04
Ave.	.....	1.185	.....	0.93

* Visible amount of alkali carried over mechanically.

[1] At the time of setting up this experiment, the mechanical difficulties attending the construction of a satisfactory and reliable rotary shaker had not been overcome, so an ordinary laboratory shaker was geared down so as to tilt the flasks back and forth at a rate of 3 complete excursions each 2 minutes.

would reveal irregularities in the method of inoculation better than would a longer period. The results reported as mgs. N per culture are shown in table VI.

Method *A*, in which the suspension was incubated 24 hours on a shaking machine, gave slightly higher results, yet growth was certain and reasonably uniform in those inoculated according to method *B*. It is rather difficult to decide between these two methods on the basis of the above experiment. In subsequent work method *A* was favored, but if for any reason it was undesirable to delay the experiment 24 hours method *B* was used. The significant thing is that growth took place in every flask seeded. The difficulty encountered in the "orientation experiments" was probably due to the fact that the suspensions were too dilute. No more difficulty was experienced in obtaining growth from the heavy suspensions used. However, some rather surprising failures of growths on plates from dilutions from the suspensions were observed. The point seemed to be that in working with *Azotobacter* heavier suspensions must be used than are needed with most other bacteria. This is possibly to be explained by their heavy slime production.

FINAL EXPERIMENTS

The few experiments which we were able to carry out after revising our methods were designed to see how far the facts would fit the hypothesis suggested above; that is, to what extent certain variations with regard to the presence of a second phase in the nutrient solution would affect the growth and development of the microörganism under study. Experiments were conducted on the following points: (1) removal of the solid phase; (2) restoring of the solid phase; (3) homogenous nutrient solutions; and (4) the action of protective colloids designed to partially or wholly prevent the flocking out of the phosphate precipitate. These experiments will now be considered in the order named.

REMOVAL OF SOLID PHASE

For the study of this point Ashby tap water dextrose[1] me-

[1] Whenever dextrose was used the culture medium was sterilized by the intermittent method.

dium was used. One portion was boiled up with calcium carbonate and then filtered through a folded filter. The filtrate was perfectly clear. Fifty-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of calcium carbonate. A parallel series was set up, using the unheated and unfiltered medium. After sterilization alternate pairs of flasks in each series were inoculated with a spiral of a 24-hour shaker culture of *Azotobacter* prepared by inoculating 10 cc. of Ashby's mannite solution in a 250-cc. Erlenmeyer flask with a spiral of a 72-hour growth of F_{10} on Ashby soil extract agar.

One-half of each series was placed on the rotary shaker and one-half on the shelf near by. The whole experiment was carried out in the warm room, the temperature of which during this particular period was very erratic. The first two days

TABLE VII

SERIES A—SOLID PHASE PRESENT

No.	Treatment	Condition at close	Nitrogen (mgs.)
		Shaker	
1	Check	Clear	0.62
2	Check	Clear	0.59
3	Inoc.	Strong turbidity, no floccules	5.10
4	Inoc.	Strong turbidity, floccules and slight pigment	5.40
			Residual sugar (mgs.)
5	Check	Clear	848.
6	Check	Clear	839.
7	Inoc.	Same as No. 4	0.0
8	Inoc.	Same as No. 4	0.0
		Shelf	
			Nitrogen (mgs.)
9	Check	Clear	0.72
10	Check	Clear	0.65
11	Inoc.	Good turbidity, some floccules	3.34
12	Inoc.	Good turbidity, some floccules	2.97
			Residual sugar (mgs.)
13	Check	Clear	820.
14	Check	Clear	825.
15	Inoc.	Same as Nos. 11 and 12	449.
16	Inoc.	Same as Nos. 11 and 12	559.

the temperature was 23–25° C., then 28–30° for 3 days, while for the last 5 days of the 10-day incubation period it was 35–37°. The rotating machine[1] was revolved once in 24 seconds. After 10 days one-half of the flasks of the shaker set and of the shelf set were subjected to nitrogen analyses, and the other half to sugar determinations. The results appear in tables VII and VIII.

TABLE VIII

SERIES B—SOLID PHASE REMOVED

No.	Treatment	Condition at close	Nitrogen (mgs.)
		Shaker	
17	Check	Clear	0.42
18	Check	Clear	0.42
19	Inoc.	Clear	0.41
20	Inoc.	Faint turbidity	0.68
			Residual sugar (mgs.)
21	Check	Clear	...*
22	Check	Clear	...*
23	Inoc.	Clear	881.
24	Inoc.	Faint turbidity	600.
		Shelf	
			Nitrogen (mgs.)
25	Check	Clear	0.42
26	Check	Clear	Lost
27	Inoc.	Faint turbidity	1.11
28	Inoc.	Faint turbidity	1.75
			Residual sugar (mgs.)
29	Check	Clear	959.
30	Check	Clear	945.
31	Inoc.	Clear	933.
32	Inoc.	Faint turbidity	914.

* By mistake these cultures were subjected to nitrogen analyses. Nos. 21 and 22 contained 0.393 and 0.418 mgs. N, respectively.

This experiment brings out very clearly the effect of removing the precipitated phosphates from Ashby's solution. The medium from which this precipitate has been removed is very poorly suited to the support of this microörganism. It seems that the shaker is detrimental with this medium, while it is distinctly beneficial when the precipitate of phosphates is

[1] Constructed similar to the one used by Bonazzi, *loc. cit.*

present. The variation in the growths in different flasks shows the undesirability of making a sugar determination on one flask and a nitrogen determination on its duplicate. The sugar determinations above are comparatively inaccurate, owing to the fact that only $\frac{1}{20}$ of the solution was taken for analysis, hence computations of nitrogen fixed per gram of dextrose consumed have not been made.

RESTORATION OF SOLID PHASE

An experiment was next conducted on adding the solid substances, $CaCO_3$ and $CA_3\ (PO_4)_2$, alone and in combination, to filtered (i. e., filtered subsequent to heating to boiling in the presence of $CaCO_3$) Ashby's tap water solution. The culture medium was therefore directly comparable to that used in obtaining the data reported in tables VII and VIII. Twenty-five-cc. portions of this perfectly clear solution were pipetted into 700-cc. flasks and a drop of 0.5 ferric chloride added to each. Calcium carbonate and calcium phosphate were added to certain of the flasks according to the plan shown in table IX, which contains also the results of the experiment. The flasks were inoculated at the same time and from the same suspension as used in the previous experiment. All flasks were incubated on the rotating machine for 10 days.

TABLE IX

No.	Material added to culture medium	Treatment	Residual glucose (mgs.)	Nitrogen (mgs.)
45	None	Inoc.	294.1	...
46	None	Inoc.	215.4	...
47	$CaCO_3$	Inoc.	181.7	...
48	$CaCO_3$	Inoc.	448.8	...
49	$Ca_3(PO_4)_2$	Inoc.	409.0	0.45
50	$Ca_3(PO_4)_2$	Inoc.	562.0*	0.44
51	$CaCO_3$, $Ca_3(PO_4)_2$	Inoc.	00.0	2.16
52	$CaCO_3$, $Ca_3(PO_4)_2$	Inoc.	00.0	2.14
53	$CaCO_3$, $Ca_3(PO_4)_2$	Sterile	448.0	0.19
54	$CaCO_3$, $Ca_3(PO_4)_2$	Sterile	450.5	0.16

* Probably an analytical error.

Owing to an accident in the introduction of the alkali into the Kjeldahl flasks to the set of 4 cultures, 45–48, these nitrogen determinations were lost. In spite of these irregularities the experiment shows undoubted benefit resulting from the addition of tricalcium phosphate, the same material filtered off in experiment 1 above. It is interesting to note that only when both the carbonate and the phosphate of calcium were added was good growth obtained.

MEDIA WHICH FORMED NO PRECIPITATE

Medium of Löhnis and Smith.—Löhnis and Smith ('16, p. 686) state that a medium of the following composition is excellent for supporting the growth of *Azotobacter* and remains perfectly clear:

Dextrose	20	gms.
Dipotassium phosphate[1]	0.2	gm.
Sodium chloride	0.2	gm.
Magnesium sulphate	0.2	gm.
Calcium sulphate	0.1	gm.
10% ferric chloride	2	drops
Distilled water	1000	cc.

This medium is essentially Ashby's solution, hence we felt sure it would yield a precipitate. When the above materials were dissolved in the cold the solution was almost but not quite clear. On heating to boiling a slight flocculent precipitate formed. After the solution had cooled this precipitate was filtered off and 25-cc. portions of the perfectly clear filtrate pipetted into 1000-cc. Erlenmeyer flasks. Two series of flasks of 6 each were prepared, the one series receiving a pinch of calcium carbonate per flask, the other not. The method of inoculation was the same as that used in the two previous experiments, the same suspension being used. Two flasks of each series were placed on the rotator, and the remaining ones on the shelf near by. The incubation conditions were the same as in previous experiments. The results are given in table x.

[1] Löhnis and Smith used monopotassium phosphate neutralized to phenolphthalein with sodium hydroxide.

TABLE X

AZOTOBACTER DEVELOPMENT IN FILTERED LÖHNIS AND SMITH'S MEDIUM

	No.	Treatment	Glucose (mgs.)	Nitrogen (mgs.)
Shelf	55	Check	464.	.42
	56	Check	452.	.34
	57	Inoc.	486.	.41
	58	Inoc.	251.	.34
Shaker	59	Inoc.	530.	.39
	60	Inoc.	524.	.37
Same conditions except $CaCO_3$ added				
Shelf	61	Check	530.	.32
	62	Check	516.	.30
	63	Inoc.	339.	.75
	64	Inoc.	385.	.90
Shaker	65	Inoc.	Lost but made abundant growth	...
	66	Inoc.	328.	.88

The clear filtered medium is very poor for the growth of *Azotobacter;* indeed there is no evidence that growth took place. When calcium carbonate is added growth is better. The fact that growth takes place in this filtered medium, whereas it failed in our first experiment, is probably due to all phosphates not being removed by the method of precipitation in this experiment, whereas they were in the first one.

Glycerolphosphate medium.—In order to prepare a medium which would remain clear and from which the phosphates would not be precipitated by heating in presence of $CaCO_3$, an organic phosphate was used. Calcium glycerolphosphate seemed to be the most promising, since it is soluble in water and does not form a precipitate with any of the salts used in Ashby's solution. Twenty-four hundredths gm. of this salt carries essentially the same amount of phosphorus as does .2 gm. K_2HPO_4 and a little more calcium than does .1 gm. $CaSO_4 . 2H_2O$. Hence this amount of calcium glycerolphosphate added to the medium supplies as much phosphorus and calcium to the culture medium as is contained in Ashby's solution. Since the molar weights of K_2HPO_4 and K_2SO_4 are practically equal, .2 gm. of K_2SO_4 will carry the same amount

of potassium as .2 gm. K_2HPO_4. The following medium was therefore prepared:

Dextrose	20	gms.
Calcium glycerolphosphate	0.2	gm.
Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Potassium sulphate	0.2	gm.
Distilled water	1000	cc.
10% ferric chloride	3	drops

No precipitate formed on heating the medium. It was filtered, however, to remove the few particles of foreign material which most likely were introduced in the dextrose. The perfectly clear medium then was found to give a P_H value of about 6.7 as measured with the aid of standards and buffer solutions recommended by Clark and Lubs ('17). Duplicate 5-cc. portions were titrated with N/50 alkali and phenolphthalein and the computed amount (9.00 cc.) of N/50 alkali necessary to bring an aliquot of the medium to P_H 8. After the addition of this amount of alkali the P_H value was found to be a trifle less than 8.

Fifty-cc. portions of this adjusted medium were then pipetted into each of twelve 700-cc. "Nonsol" Erlenmeyers. To 6 of these was then added a pinch of calcium carbonate. After sterilization by the intermittent method the cultures were incubated for 7 days at 28–30° C. The plan of the experiment is shown in table XI. The inoculum was from a 24-hour 10-cc. mannite shaker culture prepared by heavy inoculation from F_{17}, 7 days old. One spiral of this culture was used in seeding each flask. The results appear in table XI.

The results indicate that the phosphorus in glycerolphosphates is to some extent available for the growth of *Azotobacter,* although it is barely possible that the glycerolphosphate may have hydrolyzed in the faintly alkaline solutions during sterilization. The medium, although adjusted in reaction to practically P_H 8, was not suitable for growth unless $CaCO_3$ was added, practically no growth taking place in the absence of $CaCO_3$. The rotator proved beneficial in the presence of calcium carbonate.

TABLE XI

AZOTOBACTER DEVELOPMENT IN THE PRESENCE OF GLYCEROLPHOSPHATE

	No.	Treatment	Base added	Cont. of culture at end	
				Sugar (mgs.)	Nitrogen (mgs.)
Shelf	67	Check	0	910.	0.16
	68	Check	0	914.	0.16
	69	Inoc.	0	913.	0.10
	70	Inoc.	0	912.	0.13
Shaker	71	Inoc.	0	908.	0.08
	72	Inoc.	0	908.	0.10
Shelf	73*	Check	$CaCO_3$	901.	0.09
	74*	Check	$CaCO_3$	904.	0.22
	75	Inoc.	$CaCO_3$	848.	0.67
	76	Inoc.	$CaCO_3$	820.	0.99
Shaker	77	Inoc.	$CaCO_3$	275.	1.88
	78	Inoc.	$CaCO_3$	268.	2.05

* 500-cc. flasks.

ACTION OF PROTECTIVE COLLOIDS

If now one of the effects of mechanical agitation is to hasten the solubility of phosphates, it ought to be possible to replace this action in part by the use of protective colloids; that is, the colloid, by preventing the complete flocking out of these compounds, would cause a greater surface to be exposed to the action of the solvent. Agar naturally suggested itself as a possible protective colloid, and its function as such was studied in two ways: (a) in solid media, and (b) in filtered and non-filtered liquid media.

Solid nutrient agars.—Two agars were prepared from purest chemicals obtainable and redistilled water, and these compared with the regular nutrient agar. In the case of the one agar the phosphates were allowed to precipitate before the agar was added. In the case of the second medium one half the agar was added to a solution containing the calcium and magnesium salts, the other half to a solution containing the phosphate. The exact procedures were as follows:

Agar I (phosphates allowed to precipitate before the addition of agar).

Solution A

Mannite	20	gms.
Dipotassium phosphate	0.2	gm.
Double distilled water	500	cc.

Solution B

Magnesium sulphate	0.2	gm.
Sodium chloride	0.2	gm.
Calcium sulphate	0.1	gm.
Double distilled water	500	cc.

After all the salts were dissolved the two solutions were mixed, 2 drops of 10 per cent solution of $FeCl_3$ and a pinch of $CaCO_3$ added, and the whole heated to boiling to effect complete precipitation of the phosphates. Seven and one-half grams of Bacto agar were then added and dissolved with the aid of the autoclave. The solution was thoroughly stirred while hot and then filtered, tubed, and again autoclaved.

Agar II (phosphates allowed to precipitate after the addition of agar).

Solutions *A* and *B* were prepared exactly as above, and then 3.25 gms. agar dissolved in each, the solutions filtered, and then united, tubed, and autoclaved.

Agar III. This was the regular modified Ashby soil extract agar described above.

In tubing these agars 10-cc. portions were placed in Jena test-tubes containing a pinch of $CaCO_3$. Three slants of each agar were inoculated on the same date with triple strokes from a 72-hour culture of the F_4 generation. The results are shown in the following summary:

TABLE XII

SUMMARY OF GROWTH ON AGARS

Agar no.	Growth after			
	24 hours	72 hours	6 days	10 days
I	Faint growths in 2 tubes, doubtful in 3rd	Raised streaks; fair growths, as compared with No. II distinctly less	Slimy, rather thin streaks; only one tube shows spreading; growths distinctly less than in No. II	Growths somewhat wrinkled and showing some pigmentation; growths slightly less than in No. II

Agar no.	Growth after			
	24 hours	72 hours	6 days	10 days
II	Faint growths	Pronounced, raised, smooth, slimy streaks	Growths somewhat flat and spreading, becoming dry and slightly wrinkled, dry portions becoming brownish	Good growths now dry and wrinkled; pigment production quite marked
III	Slight growths	Abundant rather flat growths, apparently best in whole series	Abundant, spreading growths; streaks quite largely run together; considerable invasion and collection of slime at base of slant	Abundant, flat, wrinkled growths; some pigment production; growths slightly heavier than in No. II

The following general conclusions may be drawn from the above experiments with different agars: Agar No. I produces the poorest growth and No. III the best; agar No. II produces a growth distinctly better than No. I and almost as good as that on No. III.

Nutrient solutions.—In these experiments only enough agar was added to make the solution slightly viscous. Only one-tenth the amount of agar was used, i. e., 1.5 gms. per liter. Now, if this amount of agar functions as a protective colloid the precipitated phosphate should pass through the filter quite largely. That being true, then the effect of filtering the medium reported above (pp. 31–32) would largely disappear. To see whether any such action could be detected a modified Ashby medium was prepared, using .15 per cent agar as a protective colloid, and filtered and unfiltered portions tested with and without mechanical agitation. A modified Kaserer's solution was tested in a similar manner.

The modified Ashby solution was prepared as described above for the use of agar as a protective colloid, except that 0.75 gm. instead of 7.5 gms. was added to each of the solutions corresponding to solutions *A* and *B*. The two solutions were mixed, well stirred up with $CaCO_3$, heated for 30 minutes in the autoclave, and then a portion of the preparation filtered.

Twenty-five-cc. portions of the filtered and unfiltered medium were then placed in 1000-cc. "Nonsol" Erlenmeyer flasks containing a pinch of $CaCO_3$, plugged, capped with beakers, and autoclaved. The flasks were inoculated according to method *B* described previously (p. 27), the suspension being prepared from a 6-day-old slant of the F_{18} culture. The cultures were incubated for 10 days at 28–30° C. Part of the flasks were placed on the mechanical shaker and part were kept on the shelf near by. The plan and results of the experiment are shown in table XIII.

TABLE XIII

MODIFIED ASHBY'S MEDIUM WITH .15 PER CENT AGAR AS PROTECTIVE COLLOID

	No.	Treatment	Nitrogen (mgs.)
		Unfiltered	
Shelf	103*	Check	0.17
	104*	Check	0.13
	105	Inoc.	1.57
	106	Inoc.	1.70
Shaker	107	Inoc.	1.16
	108	Inoc.	1.39
		Filtered	
Shelf	79	Check	0.16
	80	Check	0.12
	81	Inoc.	2.45
	82	Inoc.	2.39
Shaker	83	Inoc.	1.94
	84	Inoc.	2.30

* 300-cc. flasks used.

A duplicate experiment was carried out simultaneously, with all conditions the same except that no agar was used in the nutrient medium. The results appear in table XIV.

The results show that when a small amount of agar is added to a medium in such a way that it may act as a protective colloid, this medium then is not affected injuriously by filtering. On the other hand, if the agar be omitted, the filtered medium is distinctly inferior to the unfiltered. Moreover, in the presence of the agar the shaker is apparently of no benefit to the growth of the microörganisms.

TABLE XIV
MODIFIED ASHBY'S SOLUTION WITHOUT AGAR

	No.	Treatment	Nitrogen (mgs.)
		Unfiltered	
Shelf	109*	Check	.07
	110*	Check	.03
	111	Inoc.	1.18
	112	Inoc.	0.98
Shaker	113	Inoc.	1.23
	114	Inoc.	1.31
		Filtered	
Shelf	85	Check	0.16
	86	Check	0.08
	87	Inoc.	1.24
	88	Inoc.	0.24
Shaker	89	Inoc.	0.69
	90	Inoc.	0.45

* 300-cc. flasks used.

In a similar experiment using Kaserer's medium 2 per cent mannite was used as the energy source instead of the 1 per cent dextrose employed by Kaserer. Furthermore, the potassium silicate which we had available was so strongly alkaline that the medium had to be partially neutralized after this material was added. The medium was prepared as follows: One gm. $Al_2(SO_4)_3$ and 0.25 gm. of $FeCl_3$ were dissolved in approximately 350 cc. of distilled water, the solution heated, and the Fe and Al precipitated with Na_2HPO_4 solution. The precipitate was thrown down by means of a centrifuge, decanted, washed once by the same process, and then suspended in 700 cc. water containing 15 cc. of 10 per cent potassium silicate solution. The suspension was then strongly alkaline, and the required amount of N/10 acid as determined by titration was added to adjust the reaction to P_H 8, the volume then made to 1 liter, distributed in bottles, and shaken on a machine for 4 hours. Complete solution was not effected. There was then added to the 1-liter portion:

Mannite	20 gms.
Calcium sulphate	.1 gm.
Manganese sulphate	.1 gm.
Magnesium sulphate	.1 gm.
Sodium chloride	.1 gm.

This suspension was then heated, and approximately one-half of it filtered through ordinary filter paper.

Twenty-five-cc. portions of the filtered and of the unfiltered medium were distributed into 1000-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of $CaCO_3$. Inoculations were made as described above from the same suspension and on the same date. Incubation was also under the same conditions and date. The results are reported in table xv.

TABLE XV

MODIFIED KASERER'S SOLUTION

	No.	Treatment	Nitrogen (mgs.)
Unfiltered			
Shelf	115*	Check	0.11
	116*	Check	0.05
	117	Inoc.	0.94
	118	Inoc.	0.95
Shaker	119	Inoc.	2.33
	120	Inoc.	2.40
Filtered			
Shelf	91*	Check	0.07
	92	Check	0.06
	93	Inoc.	1.15
	94	Inoc.	1.54
Shaker	95	Inoc.	2.62
	96	Inoc.	2.72

* 300-cc. Erlenmeyer flasks used.

From the above data it appears that the filtered solution is fully as good as the unfiltered, and that mechanical stirring of the cultures is beneficial to both media. This indicates that both the filtered and the unfiltered media are poorly buffered and that the mechanical action of the shaker assists in the maintenance of the proper H ion concentration by hastening the solution of the calcium carbonate.

An attempt was made to conduct an experiment similar to the above with filtered and unfiltered solutions containing Remy and Rösing's colloidal ferric oxide, but the solution precipitated completely on heating, hence the results of the experiment were without significance.

General Conclusions

The experimental work reported in this paper suggests that some of the markedly beneficial results observed in cultural solutions by different workers are associated with phosphorus nutrition of the organism and with maintenance of proper reaction of the medium. The experiments above on removal and restoration of the precipitate and on the use of glycerolphosphate, and those with protective colloids are suggestive, but do not yield the final proof of the mechanism of increased growth. The beneficial effect of the agar might be explained from the viewpoint of Kaserer, i. e., by the presence of certain nutrients in the agar, but this explanation seems less plausible than that of its action as a protective colloid.

Many experimental difficulties stand in the way of proper development of this interesting field of inquiry. Especially is this true in dealing with colloids. It is often difficult to duplicate the work of another investigator in the field of colloid chemistry, and this point is well illustrated by the contradictory results reported above with colloidal hydrated ferric oxide, in which case we were unable to duplicate even our own results. Moreover, the method of measuring growth at the end of a short incubation period, as has been done in the work reported in this paper, is wholly inadequate to permit a rigid examination of the results of different conditions. The method used by Bonazzi with the nitrite-producing bacteria, of repeatedly renewing the energy supply and measuring the products of growth, is far superior. If some such method could be used with *Azotobacter* a more reliable picture of the growth processes could be obtained.

The discussion of the arguments for and against the above theories might be extended greatly, yet this hardly seems to us worth while just at present, especially in view of the paucity of rigid experimental data. The working hypothesis suggested above may be of some help in the development of experimental work, and if subsequent experiments show it to be unsound it should be discarded. At present, however, it seems that there is fully as much in support of it as of the other

theories, and it has the advantage of being less roundabout; that is, the need of phosphates and the avoidance of an acid reaction are requirements of the culture medium, known beyond any doubt. It seems that the various ramifications of these *known* factors must be studied in detail before speculations in regard to "auximones" and "rare nutrients" be entered into widely.

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Explanation of Plate

PLATE 1

Photomicrographs of Azotobacter chroococcum, × 1170. Culture No. 5 from liquid medium, others from agar slants.

Fig. 1. A 12-hour-old culture stained with dilute aqueous methylene blue.

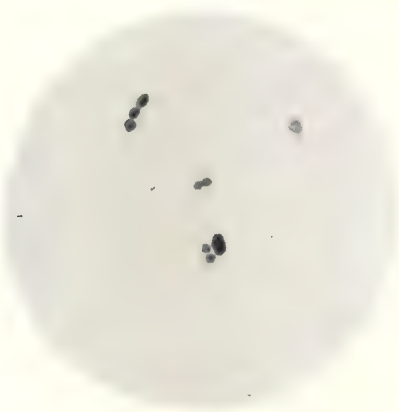
Fig. 2. Same, stained with carbol gentian violet.

Fig. 3. A 60-hour-old culture, methylene blue.

Fig. 4. Same, carbol gentian violet.

Fig. 5. A 5-day-old culture, methylene blue.

Fig. 6. A 15-day-old culture, carbol gentian violet.



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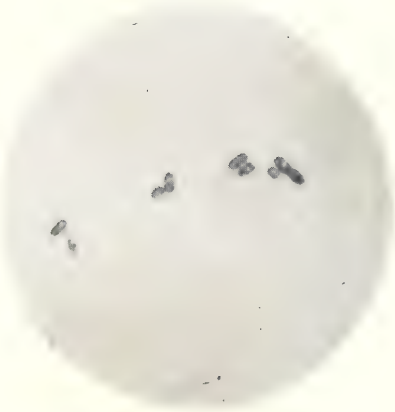

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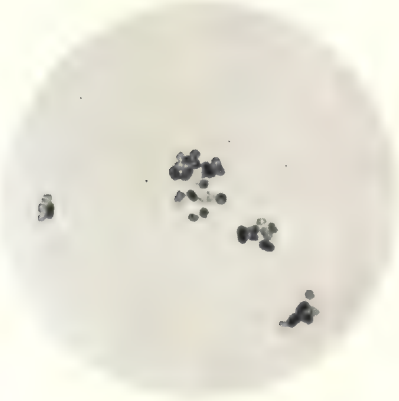

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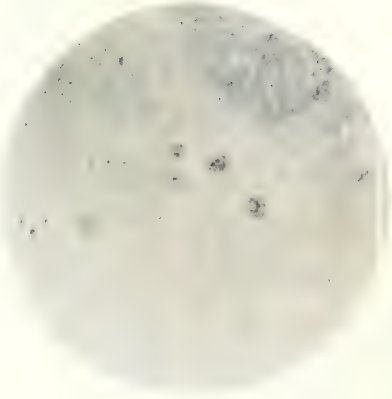

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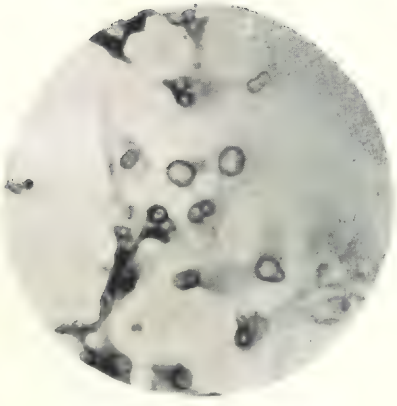

6

ALLEN—AZOTOBACTER CHROOCOCCUM

AN ALL-GLASS NITROGEN APPARATUS

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The necessity of refined analytical methods is apparent to any one who has given serious consideration to the problem or problems in certain phases of plant metabolism. This need is especially felt in the problems of nitrogen fixation by the lower forms of plant life. Helpful suggestions may be obtained from the nitrogen methods used in the field of animal biochemistry, yet these methods are usually not directly applicable without more or less revision with a view to obtaining greater accuracy even at the expense of convenience and ease of manipulation. This is illustrated, for instance, by the nitrogen method proposed by Davis[1] while working on the problem of nitrogen fixation by fungi in the laboratory of Professor Duggar. This method may be considered as intermediate, in general features, between the "micro" methods of Folin and the standard methods employing a bank of block tin stills and necessary additional devices.

Continuance of the experimentation described by one of us[2] in this laboratory and in the Wooster laboratory gave rise to an apparatus which we feel merits recommendation to other workers in similar fields. Its features are: (1) elimination of rubber stoppers and connection; (2) efficient scrubbing of the entrained alkali from the steam; and (3) the use of Pyrex glass, which does not yield an appreciable amount of alkali to steam or boiling solutions.[3]

[1] Davis, A. R. A note on the adaptability of the Folin micro-Kjeldahl apparatus for plant work. Ann. Mo. Bot. Gard. **3**: 407–412. *pl.* 7. 1916.

[2] Allen, E. R. Some conditions affecting the growth and activities of Azotobacter chroococcum. Ann. Mo. Bot. Gard. **6**: 1–44. *pl. 1. f. 1–2.* 1919.

[3] Davisson, B. S. Ammonia and nitric nitrogen determinations in soil extracts and physiological solutions. Jour. Ind. and Eng. Chem. **10**: 600–605. *f. 1–3.* 1918.

The apparatus is shown in pl. 2, which is practically self-explanatory. All parts except the condenser jacket *G* are made from Pyrex glass, which may be worked with an oxy-illuminating gas flame. The bulb *A* is conveniently made from a 200-cc. flask, the neck being drawn down and sealed to the condenser tube *F*. The tip of the curved tube in bulb *A* is perforated by several holes at its lower point. Tube *C* is attached to the 500-cc. Kjeldahl flask *E* by a ground joint at *D*.

One objection to the apparatus in this form is its rigidity, which, on shaking to mix the alkali, in the Kjeldahl procedure, renders the likelihood of breakage high. To overcome this we have used a rubber joint at *B*, which does not appear to vitiate the results if the glass tubes are fitted closely end to end. An extreme case was taken to test this fault and the general efficiency of the apparatus. Solutions of N/100 acid and alkali were prepared and carefully standardized. A dilute solution of ammonium hydroxide was carefully titrated against the solutions, using methyl red as the indicator. Successive equal portions of this ammonium hydroxide solution were distilled in the apparatus and the distillate titrated with the above-described solutions. Among the following data those results indicated by an asterisk were obtained in an apparatus with a close rubber joint at *K*.

Nitrogen taken 0.103 mgs.

N found (mgs.)	Error (mgs.)
0.101	–0.002
0.105	0.002
0.103	0.000*
0.101	–0.002
0.098	–0.005*
0.103	0.000
0.099	0.004
0.098	–0.005*
0.105	0.002
0.105	0.002*
Average 0.102	Average deviation 0.0024

Scrubbing bulb *A* effectively removes the alkali entrained in the vapor when distillations are made over strongly alka-

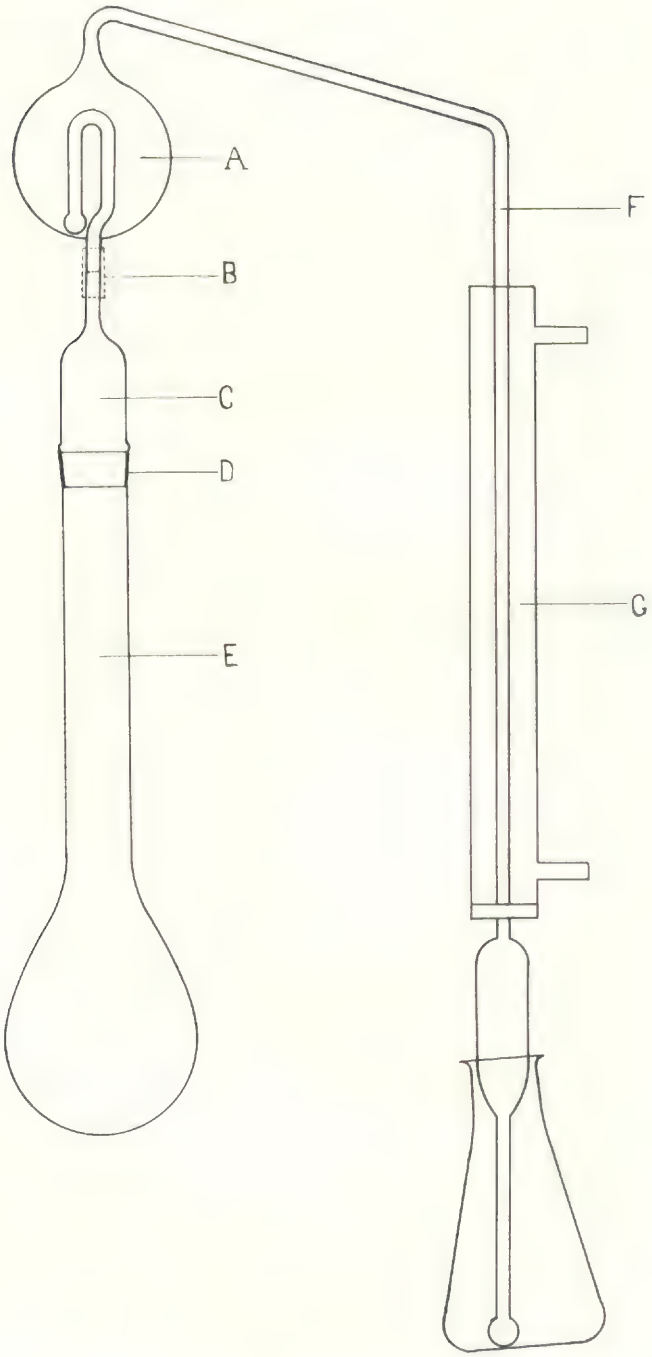
line solutions. The surface of the bulb is large enough to provide sufficient condensation to form a water trap, through which fixed alkali will not pass. This point was tested by an experiment in which conditions were also purposely extreme. There were placed 100 cc. of concentrated alkali and 100 cc. of nitrogen-free water in the distilling flask and zinc added, just as in the case of the regular nitrogen determinations. The distillate was collected in three portions of about 80 cc. each, and in no fraction was there sufficient alkali to be detected with N/100 acid.

The requirements as to quality of joint at *D* are not so exacting as in the case of joints for ether extraction and similar apparatus, for the reason that a safe connection may be made with the aid of a water seal, using a joint less close-fitting than might otherwise be demanded. For this reason we hope the manufacturer will eventually be able to make the flasks interchangeable on different pieces of apparatus. This point is under consideration at the present time.

Explanation of Plate

PLATE 2

An all-glass nitrogen apparatus. (See p. 46 for explanation.)



ALLEN AND DAVISSON—NITROGEN APPARATUS

ARCANGELIELLA, GYMNOMYCES, AND MACOWANITES IN NORTH AMERICA

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ARCANGELIELLA

Arcangeliella Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: 117–128. 1900; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 255–256. 1902.

The type species of the genus is *Arcangeliella Borziana* Cavara.

Fructifications gregarious, hypogaeous or emergent, fleshy, lactiferous; peridium thin, separable with difficulty, extending to the base in young specimens but evanescent and disappearing below at maturity; columella simple or branched, often extending to the peridium above; base more or less sterile, usually attenuated and leading to rhizomorphs, generally lactiferous; gleba fragile, lactiferous; cavities minute, irregular, radiating more or less from the columella and base; basidia 2–4-spored; cystidia present; spores globose to ellipsoidal, echinulate to verrucose, tinted.

This is a distinct genus which is closely related to *Macowanites* Kalchbr. The description has been emended here to include some variation in characters which evidently Cavara did not have the opportunity to observe in the type species and which are necessary to include *Arcangeliella caudata* and *A. Soderstromii.*

1. Arcangeliella caudata Zeller & Dodge, sp. nov.

Fructificationes globosae, base attenuata, superne complanatae aut piriformes, "dilute brunneae vel saturatius vel fusco-rubideae, area subalbida parva inferne prope stipite excepta" (Gardner), "mummy-brown" vel "clove-brown" (Ridgway) superne, "clay-color" vel "olive-brown" (Ridgway) inferne servatae, 0.8–2 cm. diametro,

superficie villosa; peridium 200–300 μ crassitudine superne tenuissimum vel absens inferne, "sepia" (Ridgway) sub lente, radialibus hyphis septatis, perpendicularibus superficiei fructificationis, pseudoparenchyma faciens cuius cellulae 9–10×11–13 μ, septis hypharum constrictis, ex peridio labuntur et globosae vel oblongae, conidiiformes fiunt; basis sterilis attenuata, hyphis hyalinis, septatis, 3–5 μ diametro confecta; ductus lactiferi numerosi ad basim attenuatam, septati, 6–8 μ crassitudine; rhizomorphi pseudoparenchymate, brunnei, multis ductibus lactiferis muniti; columella variabilis, inconspicua vel percurrens aut ramosa, ab base non distincta, eodemque colore, ductibus lactiferis paucis; gleba carnosa, "Isabella-color" vel "brownish olive" (Ridgway), inferne aperta maturitate; locelli parvi et irregulares, ex base et columella radiantes; septa hyalina, hyphis hyalinis laxe implexis, ductibus lactiferis paucis, 50–65 μ crassitudine; cystidia hyalina, magna, clavata; paraphyses cylindrici, obtusi, hyalini, septati, 19–20×4–5 μ; basidia hyalina, tenua, clavata, bi- vel tetraspora, 24–26×9–13 μ, sterigmatibus brevibus, 3–6 μ longitudine; sporae ovatae vel ellipsoideae, uno cum vacuolo magno, pedicellatae, "yellow ocher" vel "ochraceous tawny" (Ridgway), 12–14.5×9–11.5 μ, exosporio crasso, verrucoso-rugoso.

Habitat in foliis putridis *Quercus agrifoliae*. California. Novembri.

Type: in Univ. Cal. Herb., Zeller Herb., and Dodge Herb.

Fructifications globose, with attenuate base and flattened or plane above, some quite pyriform, "varying from light brown to a dark yellowish brown or maroon except on a limited area on the under side next to the very short stipe which is almost white" (Gardner), mummy-brown to clove-brown above and clay-color to olive-brown below (in alcohol), 0.8–2 cm. in diameter, surface velvety; peridium 200–300 μ thick above, very thin or wanting below, sepia under the microscope, composed of radial, septate hyphae perpendicular to the surface, forming pseudoparenchymatous tissue having cells about 9–10×11–13 μ, the septa of the hyphae becoming constricted and finally sloughing off globose to oblong conidia-like cells from the surface of the peridium; base sterile, composed of septate, hyaline hyphae 3–5 μ in diameter, with lactiferous ducts more numerous towards the attenuate point which leads to a heavy, branched rhizomorph; lactiferous ducts of base 6–8 μ broad; rhizomorphs pseudoparenchymatous, brown, supplied with numerous lactiferous ducts; columella variable from inconspicuous to percurrent, extending to the peridium above, sometimes with

lateral branches, concolorous and continuous with the base, the few lactiferous ducts smaller than in the base; gleba fleshy, Isabella-color to brownish olive, exposed near the base in older specimens; cavities small and irregular, somewhat radiating from the base and columella; septa hyaline, consisting of loosely interwoven, hyaline hyphae, few lactiferous ducts, 50–65 μ broad; cystidia hyaline, large, clavate; paraphyses cylindrical, obtuse, hyaline, septate, 19–20$\times$4–5 μ; basidia hyaline, slender, clavate, 2–4-spored, 24–26$\times$9–13 μ; sterigmata short, stout, 3–6 μ long; spores mostly ovate to ellipsoid, one large vacuole, exospore thick, verrucose-rugose, pedicellate, yellow ocher to ochraceous-tawny, 12–14.5$\times$9–11.5 μ.

In leaf mould of *Quercus agrifolia*. California. November.

The characters of the spores, columella, and peridium distinguish *Arcangeliella caudata* from the two other species

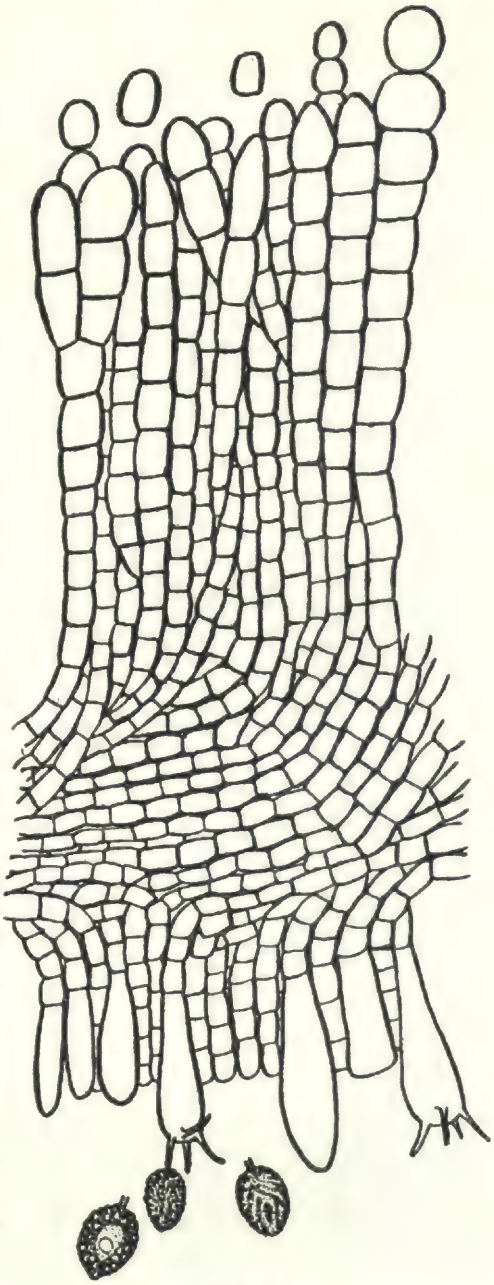

Fig. 1
A. caudata.
Section of peridium and hymenium; spores. $\times$750. From type.

of the genus. The peridium is more nearly like that of *A. Borziana* than of *A. Soderstromii,* but it is much thicker than either and has long perpendicular hyphae forming a distinct, pseudoparenchymatous tissue. The conidia-like cells which are given off from the tips of these hyphae may be an unobserved character in *A. Borziana,* but probably do not occur at all in a species like *A. Soderstromii* where the peridial hyphae extend parallel with the surface. The spores of *A. caudata* are like some Hymenogaster spores and if these only are observed one would naturally put the species in *Hymenogaster.*

Specimens examined:

California: Berkeley, *N. L. Gardner,* type (in Univ. Cal. Herb., 219, 219a, 219b, and 219c, Zeller Herb., 1623, and Dodge Herb., 1249).

2. **Arcangeliella Soderstromii** (Lagerh.) Zeller & Dodge, comb. nov.

Hydnangium Soderstromii Lagerheim in Patouillard & Lagerheim, Soc. Myc. Fr. Bul. **9** : 142. 1893; Saccardo, Syll. Fung. **11** : 172. 1895.

Illustrations: Patouillard, Soc. Myc. Fr. Bul. **9** : *pl. 8. f. 6, 6a–c.*

Type: location unknown to us, but a cotype is in the Lloyd Museum.

Fructifications subglobose to pyriform, buckthorn-brown to Isabella-color, 2–3.5 cm. in diameter; sterile base attenuate, short; columella usually percurrent, confluent with the peridium above, slender, unbranched; peridium thin, 50–70 μ thick, evanescent, ochraceous-tawny, composed of slender, interwoven hyphae, with lactiferous ducts extending parallel with the surface; gleba fragile when dry, chamois to Isabella-color; cavities variable in size, radiating from the base and columella; septa 40–45 μ broad, melleus, stupose, with a few lactiferous ducts; cystidia clavate, often mucronate, 38–40$\times$8–10 μ, hyaline, guttulate; paraphyses truncate-clavate, septate, hyaline; basidia subcylindrical, hyaline, guttulate, mostly 2-spored, 40–60$\times$6–10 μ; sterigmata stout,

8–10 μ long; spores spherical, "honey-colored," echinulate, 11–15 μ in diameter, seldom pedicellate, exospore about 2 μ thick.

In soil under *Eucalyptus*. California and Ecuador. Spring and autumn.

Lagerheim states in the original description of *Hydnangium Soderstromii* that it has no cystidia, but a study of the cotype in the Lloyd Museum reveals both clavate and mucronate forms. The specimen from California has the characteristic cystidia of the Ecuador specimens and a stout, percurrent columella with few lactiferous ducts. The chief distinction between this species and *A. Borziana* and *A. caudata* is in the peridial characters.

Specimens examined:

California: Ingleside, San Francisco, *N. L. Gardner* (Univ. Cal. Herb., 209, in part, and Zeller Herb., 1643).

Ecuador: Quito, Panecillo, *G. Lagerheim,* cotype (in Lloyd Mus., 6395); *L. Mille* (in Lloyd Mus., 12127).

EXTRA-LIMITAL SPECIES

The only reported species of this genus which has not been found in North America is the type of the genus.

1. **Arcangeliella Borziana** Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: 126. 1900; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 256. 1902.

Illustrations: Cavara, Nuov. Giorn. Bot. Ital. N. S. **7**: *pl. 7. f. 1–15.*

Fructifications hypogaeous, gregarious, globose to irregular, oblong, often bilobed, 0.6–0.8×1.5–2.0 cm. in diameter, light, nearly smooth; peridium very thin, 70 μ thick, fragile, either lacking or lacerate near the base, spotted with yellow, slightly lactiferous; gleba light rose-colored, lactiferous; columella percurrent, very lactiferous; base attenuate, sterile; latex white, sweet, abundant; basidia conspicuous, strongly exerted above the blunt paraphyses; sterigmata 3–4, acicular, long; spores spheroidal to amply ellipsoidal, dilute yellowish, echinulate, 8–10 μ in diameter; cystidia conical, acute.

In fir forests. Vallombrosa, Etruria, Italy. Summer.

The original description of *Arcangeliella Borziana* has been amplified here to include some characters which Cavara gave in his discussion of the species.

GYMNOMYCES

Gymnomyces Massee & Rodway, Kew Bul. Misc. Inf. **1898**: 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16** : 249. 1902.

Type: *Gymnomyces pallidus* and *G. seminudus* were published simultaneously with no reference to type.

Fructifications globose to irregular; peridium delicately downy or silky to evanescent, or entirely wanting; columella very much branched and dendroid when present; gleba fleshy, fertile to the base, lacunose, light-colored; cavities subequal to labyrinthiform; septa not scissile, composed of branched, interwoven, hyaline hyphae; basidia hyaline, cylindrical to clavate, mostly 2-spored; spores hyaline, globose, echinulate to verrucose.

This genus is similar to *Gautieria* in that it has no persistent peridium, but is markedly different from *Gautieria* in the spore characters. *Gymnomyces* has close affinities with some species of *Hydnangium* and *Octaviania,* having very thin peridia, but the spores are hyaline.

1. **Gymnomyces Gardneri** Zeller & Dodge, sp. nov.

Fructificationes subglobosae vel irregulares, plerumque superne inferneque complanatae, 2.5×1.5×1.5 cm. diametro servatae, 1.4×0.8×0.8 cm. siccatae, "cream-color" vel "yellow ocher" (Ridgway) servatae, "tawny olive" (Ridgway) siccatae; peridium nullum; stipes non visus; columella septa crassissima simulans, dendroidea, glebam quasi in gregibus locellorum indistinctis dividens, hyphis gelatinosis hyalinis composita, "russet brown" (Ridgway) siccata; gleba "cream-color" vel "clay-color" (Ridgway) siccata, ad basim sporifera; locelli parvi, circa 0.5 mm. diametro servati, globosi vel irregulares; septa hyalina, non scissilia, 60–80 μ crassitudine; basidia hyalina, clavata, duobus cum sterigmatibus, 25–29×9–10 μ; sterigmata tenua, 6–7 μ longa; sporae globosae vel oblongae, hyalinae, 6–9.6×10–13 μ, verrucosae (reticulato-rugosae cum maxime magnificatae sint).

Habitat in terra sub foliis *Quercus agrifoliae.* California. Decembri.

Type: in Univ. Cal. Herb. and in Zeller Herb.

Fructifications subglobose to irregular, mostly flattened above and below, 2.5×1.5×1.5 cm. in diameter in alcohol, drying to 1.4×0.8×0.8 cm., cream-color to yellow ocher in alcohol, tawny olive when dry; peridium entirely lacking; no stipe on specimens examined; columella dendroid, resembling much-thickened septa, dividing the gleba into indistinct areas, consisting of quite gelatinous, hyaline hyphae, and drying to a russet brown; gleba cream-color to clay-color when dry, fertile to the base; cavities small, averaging 2 to the mm. in alcohol, globose to irregular; septa hyaline, not scissile, 60–80 μ broad; basidia hyaline, clavate, with 2 sterigmata, 25–29×9–10 μ; sterigmata slender, 6–7 μ long; spores globose to oblong, hyaline, 6–9.6×10–13 μ, verrucose (reticulate-rugose under the oil immersion).

Fig. 2
G. Gardneri.
Basidia and spores.
×750. From type.

Upon the ground under leaves of *Quercus agrifolia*. California. December.

G. Gardneri differs from *G. pallidus* and *G. seminudus* in color and spore characters and in that it has a columella. In spore characters it has nearest affinities with *G. pallidus*.

Specimens examined:

California: Alameda Co., Berkeley, *N. L. Gardner*, type (in Univ. Cal. Herb., 376, and in Zeller Herb., 1618).

EXTRA-LIMITAL SPECIES

We are including the descriptions of the extra-limital species to assist in referring material to them should these be found in North America. The descriptions are translations from the original.

1. Gymnomyces pallidus Massee & Rodway, Kew Bul. Misc. Inf. **1898**: 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16**: 249. 1902.

Type: *Rodway, 299,* in Kew Herb.

Fructifications irregularly globose, 2–4 cm. in diameter, very fragile; no distinct peridium; gleba at first white, then dirty white; sterile base obsolete, but in one specimen the base growing into a slender stem emerging from an umbilicus; glebal cavities somewhat enlarged, irregular, dirty white; septa narrow, white, not scissile; spores globose, 9–10 μ in diameter, hyaline, verrucose, often short-caudate, two to each basidium, supported on short sterigmata.

Under ground. Tasmania.

2. Gymnomyces seminudus Massee & Rodway, Kew Bul. Misc. Inf. **1898** : 125. 1898; Saccardo & Sydow in Sacc. Syll. Fung. **16** : 249–250. 1902.

Type: *Rodway, 124,* in Kew Herb.

Fructifications globose, 1.5–2.5 cm. in diameter; peridium when present delicately tomentose; gleba white, fertile to the base; glebal cavities minute, very crowded, empty, irregular; septa somewhat broad, white, not scissile; basidia subclavate; sterigmata two; spores spherical, 11–12 μ in diameter, closely echinulate, hyaline.

Emerging from the ground. Tasmania.

Gymnomyces seminudus Mass. & Rodw. is distinguished from *G. pallidus* Mass. & Rodw. by the larger, strongly and densely echinulate spores.

MACOWANITES

Macowanites Kalchbrenner in Sacc. Syll. Fung. **7** : 179. 1888; Fischer in Engler & Prantl, Die Nat. Pflanzenfam. **I.** 1** : 299–300. *f. 148.* 1899.—*Macowania* Kalchbrenner, Gardeners' Chron. N. S. **5** : 785. *f. 141.* 1876.—Not *Macowania* Oliver in Hooker, Icon. Pl. III. **1** : 49. 1870.

The type species of the genus is *Macowanites agaricinus* Kalchbrenner.

Fructifications subglobose to hemispherical, epigaeous or hypogaeous, stipitate, fleshy; peridium covering the upper surface of the fructification, thin; stipe distinct below, but may or may not reach to the peridium above as a percurrent

columella; gleba covered above, exposed and decurrent, adnate or sinuous below; cavities globose to irregular; septa homogeneous; basidia 2-spored; spores spheroidal to ovate, hyaline, tuberculate or echinulate.

This genus was first described as *Macowania* by Kalchbrenner, in 1876, but since this name was preoccupied by *Macowania* Oliver (1870), DeToni changed the name to *Macowanites,* retaining Kalchbrenner as the author.

The genus *Macowanites* Kalchbr. is an extremely close ally to the genus *Arcangeliella,* differing mainly in the absence of lactiferous ducts. The spores of *Arcangeliella* are usually ellipsoidal and tinted, while those of *Macowanites* are spherical and hyaline. This, however, does not hold for *Arcangeliella Soderstromii,* which has spherical spores.

The fact that the two known species of *Macowanites* are from such widely separated localities would indicate the probability that this genus is much more widely distributed than at present known.

1. Macowanites echinosporus Zeller & Dodge, sp. nov.

Fructificationes subglobosae vel irregulares, 1×1.5 cm. diametro, laeves, subtiliter "salmon-colored" (Gardner) recens lectae, "tawny olive" (Ridgway) servatae; peridium tenue, 90–120 μ crassitudine, in dimidio superiore fructificationis instructum, hyalina, pseudoparenchymate parallela cum superficie fructificationis confectum; stipes eodem colore, 5 mm. longitudine, 2 mm. diametro, stuposus, tenuibus hyphis hyalinis contextus; basis sterilis, proiectura conica stipitis, sed non ut columella in glebam proiciens; gleba superne tecta, inferne aperta, non decurrens sed circum stipitem sinuata, eodem colore ut peridium; locelli minuti, irregulares; septa 60–80 μ crassitudine, hyalina, pseudoparenchymate confecta, non scissilia; cystidia infrequentia, clavata, apiculata, 9–10×20–24 μ, hyalina; basidia parva, 5–8×18–22 μ, cylindrata vel clavata, bi- vel tetraspora, hyalina; sporae sphaeroideae vel late ovatae, hyalinae, appendiculatae, 6–8 μ diametro, uno cum vacuolo, minute sparsimque echinulatae.

Habitat in terra sub *Quercu agrifolia.* California. Mart.

Type: in Univ. Cal. Herb. and Zeller Herb.

Fructifications subglobose to irregular, 1×1.5 cm. in diameter, even, smooth, very delicate salmon-color (Gardner), in alcohol tawny olive; peridium thin, 90–120 μ thick, extending over the upper half of the fructification, consisting of a

hyaline pseudoparenchyma extending parallel with the surface; stipe concolorous, about 5 mm. long and 2 mm. in diameter, stupose, of fine hyaline hyphae; sterile base a conical projection of the stipe extending into the gleba but not percurrent; gleba covered above, exposed below, not decurrent but sinuate about the stipe, concolorous with the peridium; cavities minute, irregular; septa 60–80 μ broad (including hymenia), hyaline, composed of pseudoparenchymatous cells, not scissile; cystidia rarely present, clavate, apiculate, 9–10$\times$20–24 μ, hyaline; basidia small, 5–8$\times$18–22 μ, cylindrical to clavate, 2–4-spored, hyaline; spores spherical to broadly ovate, hyaline, appendaged, 6–8 μ in diameter, one large vacuole, finely and sparingly echinulate.

Fig. 3
M. echinosporus.
Basidia and spores.
×625. From type.

Hypogaeous under *Quercus agrifolia*. California. March.

Macowanites echinosporus is distinct from *M. agaricinus* in spore characters, color of the fructifications, relation of gleba to stipe, and in that the columella does not extend to the peridium above. The generic description has been emended to include these characters.

Specimen examined:

California: East Oakland, *N. L. Gardner*, type (in Univ. Cal. Herb., 402, and Zeller Herb., 1624).

EXTRA-LIMITAL SPECIES

The type species of this hitherto monotypic genus has not been found in North America, but the original description is appended for taxonomic convenience.

1. **Macowanites agaricinus** Kalchbrenner in Sacc. Syll. Fung. **7**: 179. 1888.

Macowania agaricina Kalchbr. in Gardeners' Chron. N. S. **5**: 785. 1876.

Illustrations: Kalchbrenner, Gardeners' Chron. N. S. **5**: 785. *f. 141;* Fischer in Engler & Prantl, Die Nat. Pflanzenfam. **I.** 1**: *f. 148.*

Type: probably at Kew. Fragment in N. Y. Bot. Gard. Herb.

"Peridium hemispherical, even above, dingy, of a dirty brown, produced below into a short stem-like, smooth, white process, which penetrates up to the apex of the peridium, and is surrounded above by the large cells of the hymenium, which are below much elongated and project beyond the peridium, their apertures open to the air and decurrent. Odour strong, like that of Garlic; spores rather large, globose; epispore thick, slightly tuberculate."

—Kalchbrenner.

Habitat: among *Acacia* thickets. East Somerset, South Africa.

In this work we have used as a standard for color descriptions Ridgway, 'Color Standards and Nomenclature,' Washington, D. C., 1912. In citing specimens we have given the data received with the specimens. Wherever possible the location of the specimens has been given.

In conclusion we gratefully acknowledge all who have aided in this work. We are indebted to the Missouri Botanical Garden for the use of the library and the herbarium; to Dr. E. A. Burt and Dr. J. M. Greenman for helpful suggestions; to Dr. N. L. Gardner and Dr. W. A. Setchell for extensive collections of *Hymenogastrales* from California; to Mr. C. G. Lloyd for the privileges of his herbarium; to Dr. W. A. Murrill for helpful correspondence; and to Dr. Myron R. Sanford, Middlebury College, for helpful suggestions.

THE USE OF THE COLORIMETER IN THE INDICATOR METHOD OF H ION DETERMINATION WITH BIOLOGICAL FLUIDS

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In recent years it has become essential that physiologists, bacteriologists, and biochemists generally shall be able to determine accurately and conveniently the approximate actual reaction or hydrogen ion concentration of solutions or media of various types. It is almost inconceivable that any extensive work with biological solutions, including fermentation products and culture fluids, may proceed without adequate consideration of this factor.

It is clearly recognized that as an absolute standard in the measurement of the H ion concentration of solutions one must rely upon the use of the hydrogen electrode, whether with or without the more recent developments in the way of direct-reading potentiometers. Nevertheless, the electrical or gas-chain method requires considerable physico-chemical experience and a type of apparatus not commonly available to the physiologist or bacteriologist.

To students working in the fields just mentioned and employing nutrient solutions, decoctions, plant juices, the products of fermentation, etc., the indicator method in its present standard of development makes a strong appeal. This is true because: (1) an adequate degree of accuracy is usually attainable by this means, especially if the standard solutions are occasionally checked by the electrometric method; (2) the indicator method has special application where the quantity of material available may be small and the determinations need to be made promptly, as occasions

arise; and (3) it requires no thermal bath or other supplementary apparatus except perhaps a colorimeter in the cases to be discussed later. So long as the test solutions or media employed are colorless, or practically so, the indicator method presents now no difficulties which are not readily precluded by a little experience.

Rapid advances, however, have been made during the past few years in the perfection of buffered or standard solutions of carefully determined hydrogen ion concentration with which to compare the fluids studied. The contribution made by Clark and Lubs ('17) in respect to standard solutions is of almost equal importance to the excellent choice of indicators presented by them. In view of the availability of the work of Clark and Lubs and the detailed discussion by them it is unnecessary to refer to the preparation of such standard solutions further than to emphasize the necessity for all the refinements prescribed. In the work here reported, as well as in other studies now in progress, use has also been made of the standard solutions of Sörensen ('09–'10).[1] We have found, however, that the citrate and glycocoll mixtures undergo rapid deterioration, while the thallate, phosphate, and borate mixtures are much more stable. All solutions, whether the prepared standards or the stock solutions from which these are made, should be kept in well-seasoned glassware, and, so far as possible, the same container should be employed for a particular ionic concentration. Moreover, since the introduction of the thymol, cresol, phenol, and certain benzene products, it is no longer necessary to choose a doubtful indicator from the extensive charts of the earlier investigators, such as those of Salm ('06).

The newer indicators exhibit, for the most part, brilliant color changes throughout the range of P_H values usually required; although, as subsequently emphasized, particular care is required in the case of colored test fluids both in respect to the choice of the indicator and in checking the ac-

[1] Reference is here made to Sörensen's paper in the Carlsberg Compt. rend. des Trav. rather than to the other source of this material—Biochem. Zeitschr., 1913—in view of the fact that in the former only is a correction made (at the end of the paper) for an error in stating the amount of the phosphate employed.

curacy of the determinations made near its limits of brilliancy by another indicator with slightly overlapping color change. Moreover, it is often desired to use a particular indicator at or near the limits of its usual range. Thus methyl red, extremely serviceable between P_H 4.4 and P_H 6.0, just fails to completely cover the range of certain nutrient solutions and plant juices frequently employed in the culture of fungi. The limitation, however, is really in the ability of the unaided eye to detect readily the slight differences when a certain redness (or yellowness) is approached. The difficulty of color in the medium under investigation, however, is the most serious. In our work the indicator solutions have been prepared by using the quantities recommended by Clark and Lubs in 50 per cent ethyl alcohol. These are preserved in amber dropping bottles.

When a careful technique is established the degree of accuracy sufficient for all practical purposes is assured in the examination of colorless solutions by the following procedure: Small test-tubes containing a measured quantity (usually 5 or 10 cc.) of the standard solutions are arranged in open racks provided with a white paper background. A series is prepared for each indicator employed, and the P_H values may differ by .1 or .2, depending upon the accuracy required. A definite and constant quantity of the indicator, usually 2 or 3 drops, is placed in each tube. The same quantity of the sample or test solution is placed in a similar test-tube and the indicator added as before. The samples are then compared with the various standards in a uniform light and an exact match is obtained. A characteristic of most fluids or media with which the physiologist deals is color, and this has operated in the past more or less to interfere with the correct determinations by the indicator method.

Early investigators were disturbed by the presence of color in the solutions studied, and various methods were employed to counteract this source of error. Sörensen ('09–'10) proposed a method of dealing with colored solutions, whereby the natural test solution was matched in color by means of neutral dyes used in the standard solutions, before the addition of

the indicators. Aside from being tedious, this method gave at best only a rough comparison, exhibiting obvious errors and leaving much to the personal equation or opinion of the observer.

Walpole ('10, '10[a]) introduced a logical procedure involving the use of the colored test solution as a shield to compensate for the color of the sample under observation. He arranged a simple device which when employed for H ion determination consisted of a blackened frame or support holding four glass cells in two similar columns. Each column consists of a cell surmounted by a Nesslerizing tube, and each column is illuminated from a dull white surface below, reflecting the light upward. In the one column the lower tube contains the colored test fluid or sample plus indicator, and the upper tube water; while in the other column the upper tube contains the standard solution (in that case Sörensen's), and the lower the test fluid as shield. In each column the light passes through the colored sample and through a colorless solution, either one or the other, but not both, containing the indicator. The contents of the tube with standard solution may be changed, or other cells introduced differing slightly in H ion concentration, until, on looking down through the column, an exact match is obtained. Although obviously defective optically, this simple tintometer is serviceable. The apparatus has also been used considerably for titration work.

Independently, Hurwitz, Meyer, and Ostenberg ('16) devised at about the same time another simple apparatus for the compensation of color when the indicator method of H ion determination is employed. In this, designated a comparator, the same principle as above is applied, but the stand is so formed that four test-tubes are supported vertically in pairs and in the same horizontal plane. The system thus consists (1) of one pair of tubes (in the direction of the line of vision) with the nearer tube containing the standard solution and indicator, in front of a "shield" tube containing the colored sample; while (2) the other pair of tubes consists of one tube containing the sample and indicator, shielded beyond by a tube of water or of standard solution without

indicator. The disadvantages of this instrument are practically the same as those mentioned above, but it has obvious advantages over the usual test-tube comparison.

In some studies on the nutrition of the fungi wherein a variety of plant decoctions was employed the writers experienced the usual difficulties in rapidly and accurately employing the indicator method for determining the active acidity of these media. The plant decoctions were made in accordance with our usual method, which consists in slicing the product, or cutting it into short lengths, adding the requisite amount of water, and autoclaving at 10–15 pounds pressure for one hour. The effect of this autoclaving for extraction, together with another interval of 15–30 minutes for sterilization, after filtering into flasks, is to yield a decoction which is often highly colored. Solutions prepared from rhubarb, celery, carrots, prunes, apples, mangolds, and sweet potatoes gave, as might be expected, more pronounced color than those made from sugar beets, potatoes, and green beans. In any case, after repeated sterilizations the deepened color became a source of considerable annoyance. It should be stated that this work was begun prior to 1917, so that we were not at first in possession of the newer indicators.

In any case it seemed wise to investigate the possibility of employing the colorimeter in such work. At first no reference could be found in the literature to the use of the colorimeter in that way. Nevertheless, Veley ('06), Tizard ('10), Walpole, and perhaps others had apparently, with no great amount of consistency, employed the colorimeter in the determination of the constants of indicators and in other related work. Prideaux ('17) expresses regret that all necessary conditions—referring especially to the concentration of the indicators and to whether or not a colorimeter was used —have not been carefully specified for each indicator constant, so that it might be employed in the colorimetric determination of the H ion with greater confidence. It would appear, however, that he has employed the colorimeter directly in the determination of H ion concentration, because of the following statement: "The accuracy of a colour com-

parison by eye cannot easily be brought within 0.1 in the hydrogen exponent. With a colorimeter it is perhaps possible to obtain results agreeing to 0.01, but such an accuracy is unnecessary and is not practicable in ordinary tests of acidity or titrations." Our attention, however, was not directed to this fact until after the completion of our method, and indeed only after a careful search of his book with the idea of determining whether such references could be found in related literature.

In this work a complete Kober ('17) nephelometer-colorimeter was employed, as this instrument happened to be at hand. In reality, it possesses two distinct advantages, namely, uniform and effective source of light, and protection from side illumination. It was realized that since the colorimeter was only required in the study of colored solutions, the important factor in this case was to apply effectively the method of shield solutions. This was ultimately accomplished so satisfactorily that the defects of the comparator method were entirely obviated, while all the advantages of the colorimeter were retained.

The method consisted simply in arranging for each side of the colorimeter a pair of cups slipping to a certain depth (noted later) one into the other, as shown in fig. 1. The method of procedure is then as follows: For the left-hand set, or column, water (or colorless standard solution) is used in the outer cup, and the colored test fluid plus indicator in the inner cup. After adjustment, this set is not removed from the colorimeter during an observation. In the case of the right-hand set the outer cup contains the colored test fluid, while the inner cup is for the standard solution plus indicator. This set is placed on the right for convenience, as it may be necessary to compare with the test fluid a series of standards until an exact match is obtained. A rough comparison is of course made before selecting the standard solution for comparison. In each case the column must contain an equal depth of colored test solution and of standard or colorless liquid, the indicator being in the standard in the one case and in the test solution in the other. There are no

optical difficulties, and unless the indicator combines with the test solution, the comparison may be perfect.

In order that equal depths of liquid may be examined it is only necessary to know, or gauge by suitable washers, the distance *a* to *b* in the figure; then (if the inner cup is not the exact length of the plunger) after placing the cups on the carriers they are raised until the tip of the plunger barely touches the bottom of the inner cup when the position is read on the scale. The cups are then lowered to a distance equal to the line *ab*. It is to be noted that the quantity of solution to be placed in the cups is not necessarily determinate, so long as there is at least sufficient depth in each to equal the distance *ab*. We have found that a depth of 10–15 mm. of liquid is not too great with the instrument employed, assuming that the red indicators are utilized. In order that air bubbles may not catch under the lens the inner diameter of the inner cup should be 4–5 mm. greater than the diameter of the plunger, and similarly the inner diameter of the outer cup should be correspondingly greater than the outer diameter of the inner cup. It is also evident that good optical glass should be employed for the bottoms of the cups.

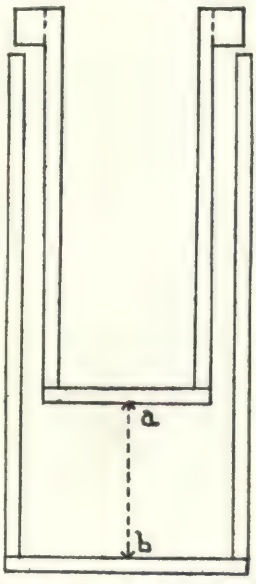

Fig. 1. Special colorimeter cups.

It is believed that the colorimeter may be employed in this work almost as rapidly as the comparator, and certainly with greater confidence and accuracy. For rapid work it is essential that one should understand the particular indicator in colored solution; likewise the effects of the quality of light employed on the color of the field, but these are minor difficulties. The red indicators, with color change red-yellow or yellow-red, have proved particularly satisfactory. In the use of these it soon became obvious that the usual P_H range of each might be considerably extended by the use of the colorimeter, but in any case the extent of the useful range is somewhat

dependent upon the intensity of color in the test fluids. With a weak beet decoction plus acid or phosphates methyl red was useful from P_H 3 to P_H 6.8, and phenol red (phenolsulphonphthalein) from P_H 6.4 to P_H 9.0.

Any refinements in the use of the simple indicator method of hydrogen ion determination should find many applications in the wide range of plant physiological studies with both lower and higher organisms. The importance of this factor of active acidity has been repeatedly urged in recent work, yet it is not receiving general consideration. In medicine the value of such determinations has gradually become apparent following the interesting development of views regarding neutrality regulation in animal fluids (compare some of the work of Henderson, '08, '09, '09[a], of Henderson and his associates, and others).

In making determinations of the hydrogen ion concentration of the blood, Levy, Rowntree, and Marriott ('15) have employed a dialysis method, used also in a study of the buffer value (Levy and Rowntree, '16) of this fluid, while a more accurate modification of the method (Marriott, '16) is used to determine the alkali reserve of the blood plasma. In plant studies the matter of neutrality regulation might seem on first thought to be of relatively little consequence, because of the diversity of reaction. The extent of the acid reserve in a general way is appreciated, but the determination of this has been largely incidental to other considerations. It would be interesting to know to what extent an acid reserve is a general characteristic of plant metabolism.

From the studies reported on animals it would appear that the protoplasm of many organisms is approximately neutral, but the indications would seem to be that plant protoplasm is often far from neutral, frequently exhibiting a relatively high acidity. It is still a question, however, to what extent the P_H determined for the juice (as a whole), representing to a large extent the contents of the vacuoles, is an index of the reaction of the protoplasm (Haas, '16). The case of certain citrus fruits is, of course, an exception, since here the more acid juice is contained in special sacs. Aside from this

instance there is still exhibited a remarkable P_H range, determined by Haas for the juices of certain higher plants to be between P_H 3.0 and P_H 7 or 8. This, moreover, is more or less comparable to the relation of certain mould fungi (notably *Penicillium italicum* and *Aspergillus niger*) to the reaction of nutrient media.

From the work here reported on the use of the colorimeter it may be concluded that (1) the difficulties involved in the approximate determination of the hydrogen ion concentration of solutions exhibiting color may be largely overcome; and (2) the useful range of certain brilliant indicators may be so considerably extended that the number of indicators employed may be materially reduced.

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TYROSIN IN THE FUNGI: CHEMISTRY AND METHODS OF STUDYING THE TYROSINASE REACTION

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Introduction

That certain fungi turn blue or black on exposure to the air and that this property is destroyed by heat has been known for many years, Pallas (1771), Bonnet (1781), Saladin (1779), and Bulliard (1791) having incidentally recorded observations of the fact. Macaire (1824), in a very extended memoir on the subject, brought out nearly all the main lines of proof of the essential features of the reactions involved, although some of his data are better explained on the theory of enzyme action which has developed since his time. Since his work many additional facts have been ascertained, as may be seen by the excellent review papers by Kastle ('10), Clark ('10, '11), and Bach ('13), as well as by others of the Geneva workers to be mentioned later. All of the work so far has dealt almost exclusively with the formation of pigment, and practically nothing is known of the actual chemical changes beyond the fact proved by Macaire that oxygen was absorbed and suggestions as to a partial oxidation of the side chain in the case of tyrosin.

This being the general situation, an attempt has been made to determine some of the chemical changes taking place in the reaction. After an extensive study of the available literature, it seemed best to attack the problem of the tyrosinase reaction first, since this enzyme is more specific and much more is known regarding the products of oxidation as they have been studied in the animal organism.

The outstanding fact in the chemistry of tyrosin is its low solubility (1:2500), which necessitates refining many of the ordinary chemical procedures in order to work with the acid

itself, not its sodium salt nor its hydrochloride. Work with the acid would enable one to work with the substrate much nearer the neutral point than would have been possible otherwise, thus avoiding interfering conditions due to hydrogen ion concentrations other than that of water.

The literature on the oxidases in general, including most of the papers on the tyrosinase reaction, has been so excellently reviewed by Behrens ('07), Kastle ('10), Clark ('10, '11), Bach ('13), Rose ('17), and probably Schweizer ('16), that there is little need to add to the reviews already published, although certain works will be discussed in some detail in connection with the methods employed and the results obtained.

I wish at this point to acknowledge indebtedness to those who have so kindly offered suggestions as the problem has developed. Thus, I wish to thank the Missouri Botanical Garden and the Medical School of Washington University for the use of library and laboratory facilities; Dr. B. M. Duggar, under whose supervision the work has been done and without whose advice it would have been difficult indeed; Dr. P. A. Shaffer, Dr. Lucien J. Morris, and Dr. E. R. Allen, all of the Medical School, for valuable suggestions regarding chemical procedures.

Methods

Extraction and precipitation of the enzyme.—Of the many methods used for the extraction of the enzyme and its separation from laccase, fractional precipitation has been most widely used (Chodat, '10, Wohlgemuth, '13). The methods previously reported by Bertrand ('96), Bach ('08, '10), Chodat and Staub ('07), Bertrand and Muttermilch ('07), von Fürth and Schneider ('01), and von Fürth and Jerusalem ('07), were tried out, but for the conditions under which I worked none proved especially satisfactory. I was unable to filter rapidly enough to prevent the inactivation of the enzyme by its long contact with the precipitant. The use of the sap before precipitation was tried, but this was not very satisfactory, since it necessitated diluting the tyrosin

and thus adding to the already great difficulties of analysis, owing to the extreme insolubility of the product. Therefore in the work with tyrosin, the dried fungous flour was added directly to the substrate, toluol added, and the mixture left to extract the enzyme and the enzyme to react with the tyrosin, a general method used by Zeller ('17). In work with phenylalanin and other amino-acids which have a much greater solubility than tyrosin, the calculated amount of fungous flour was extracted with chloroform water for 24 hours, filtered, and the filtrate mixed with an equal volume of N/250 of the amino-acid used, thus making the final concentration N/500, approximately that of a saturated solution of tyrosin, and allowing the same chemical procedures to be followed.

The fungi were brought in from the collecting trip, cut up by a vegetable slicer into pieces about 1–2 mm. thick, spread out on the table top, and allowed to dry at room temperature, either with or without an electric fan to keep the air in circulation. Some, such as *Daedalea confragosa,* which are coriaceous, were treated alternately with two or three volumes of 95 per cent alcohol and acetone until most of the water was removed from the tissues, then dehydrated with absolute alcohol and dried as above, in order to facilitate grinding by rendering the tissue more brittle. The fungous "chips," resembling the potato chips of commerce, were ground in a large mill to about the fineness of wheat bran. This was sifted through an 80-mesh sieve and the powder stored in glass bottles, which were sealed with paraffin until needed. The bran was likewise stored in bottles awaiting a chemical study. The fungi were obtained in the fall of 1916, except the material of *Polyporus sulphureus,* of which about 10 kilos fresh weight were obtained in the fall of 1917.

Methods previously employed in the study of the tyrosinase reaction.—Most of the work done on the tyrosinase reaction has been qualitative, where the enzyme has been allowed to come into contact with the substrate, and the resulting colors noted.

Five kinds of quantitative methods have been used in the study of this reaction. Von Fürth and Jerusalem ('07) have

5. *Richards' solution* (*E*)[1] which contains 0.5 gm. monobasic potassium phosphate, 4 gms. potassium nitrate, 2.5 gms. magnesium sulphate, 10 gms. ammonium nitrate, 50 gms. cane sugar, and a trace of ferrous sulphate in 1000 cc. of water.

6. *Sap from Acer saccharinum.*

The following twelve fungi were employed in the experiments: *Coniophora cerebella* Pers., *Daedalea confragosa* (Bolt.) Fr., *D. quercina* (L.) Fr., *Lentinus lepideus* Fr., *Lenzites vialis* Pk., *Merulius lacrymans* (Wulf.) Fr., *M. pinastri* (Fr.) Burt, *Pleurotus sapidus* Kalchb., *Polyporus lucidus* (Leys.) Fr., *Polystictus hirsutus* Fr., *P. versicolor* (L.) Fr., and *Trametes Peckii* Kalchb.

Methods

Twenty-five-cc. quantities of the above-mentioned solutions and decoctions were pipetted into Erlenmeyer flasks (125 cc. capacity). Duplicates of these were inoculated with each fungus after the flasks were sterilized at 15 pounds pressure for 20 minutes. Control flasks of all solutions were prepared in duplicate.

Before inoculation the fungi were all grown on agar-poured plates. The nutrient agar was made up in the following manner: To 1000 cc. of potato water (from 200 gms. of peeled and sliced potato cooked for 30 minutes in the autoclave at 15 pounds pressure) were added 20 gms. of cane sugar, 10 gms. of potassium nitrate, 5 gms. of monobasic potassium phosphate, and 20 gms. of agar. After growth of the fungi the plates were cut into small squares (about 8 mm. square) which were used as inocula. Thus the amount of agar added to the cultures introduced to all a negligible amount of nutrient materials. Similar squares of agar from uninoculated poured plates were added to all control flasks. All cultural operations were performed in a transfer room which was thoroughly steamed before each operation. There were no contaminations in the 332 cultures involved in the data discussed below.

[1] Richards, H. M. Die Beeinflussung des Wachsthums einiger Pilze durch chemische Reize. Jahrb. f. wiss. Bot. **30**: 665–688. 1897.

The cultures were incubated at room temperature (about 15–25° C.) during a period of 30 days.

The growth of the fungi which is reported in values of × in the table was determined as follows: The culture of maximum growth in the whole series was arbitrarily designated as 10 ×, no growth as 0, and intermediate amounts of growth as 1 ×, 2 ×, 3 ×, etc.

The H ion concentrations were determined in accordance with the methods devised by Clark and Lubs,[1] and are given in P_H exponents. In the table are given the P_H values of the control flasks, the P_H values of the solutions after the fungi had grown in them for thirty days, and the amount of change in the H ion concentration of the solutions brought about by the growth of the various fungi. If the change due to growth of the organism is toward the alkaline side from the true neutral point (P_H 7) it is designated as minus and if toward the acid side, plus.

Discussion

The data presented in the table bring out some interesting features concerning the comparative growth of the fungi involved. There is a decrease in the amount of growth of *Lenzites vialis, Daedalea confragosa, D. quercina, Trametes Peckii, Merulius lacrymans, Lentinus lepideus, Coniophora cerebella, Polyporus lucidus,* and *Polystictus hirsutus* when passing from the mono- to the tribasic potassium phosphate in Czapek's solution. In *Merulius pinastri* there is a slight increase in growth, in *Pleurotus sapidus* no variation, while the growth of *Polystictus versicolor* is irregular when passing in the same direction.

In Reed's solution there is a decrease in the growth quantities of all of the fungi, except *Pleurotus sapidus, Merulius lacrymans,* and *Coniophora cerebella,* when passing from the mono- to the tribasic potassium phosphate. In *Pleurotus sapidus* growth is equal in all cases, in *Merulius lacrymans* there is a steady increase, while in *Coniophora cerebella* it is irregular.

[1] Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. I. Jour. Bact. 2: 1–34. 1917.

TABLE I

GROWTH OF WOOD-DESTROYING FUNGI ON LIQUID MEDIA

Fungus		Dunham's Solution	Czapek's sol. (1) with KH_2PO_4	Czapek's sol. (2) with K_2HPO_4	Czapek's sol. (3) with K_3PO_4	Reed's sol. (1) with KH_2PO_4	Reed's sol. (2) with K_2HPO_4	Reed's sol. (3) with K_3PO_4	Maple sap	Richards' sol. (1) with KH_2PO_4	Richards' sol. (2) with K_2HPO_4	Richards' sol. (3) with K_3PO_4	Pine decoction
L. vialis	Gr.*	0	2×	2×	0	3×	2×	0	3×	5×	5×	5×	0
	P_H†	7.0	3.4	6.0	8.6	5.2	6.6	7.0	7.2	2.6	2.6	3.0	5.2
	Diff.‡	0.0	+1.8	+1.0	0.0	0.0	0.0	0.0	0.0	+2.2	+3.2	+3.2	+0.2
M. pinastri	Gr.	4×	9×	9×	10×	3×	2×	1×	8×	3×	5×	8×	0
	P_H	7.6	5.8	7.4	7.4	6.2	6.8	7.0	6.2	5.4	6.8	6.6	5.4
	Diff.	−0.6	−0.6	−0.4	+1.2	−1.0	−0.2	0.0	+1.0	−0.6	−1.0	−0.4	0.0
D. quercina	Gr.	0	6×	0	0	3×	0	0	0	6×	5×	1×	0
	P_H	7.0	3.2	7.0	8.6	5.2	6.6	7.0	7.0	2.6	2.8	5.4	5.4
	Diff.	0.0	+2.0	0.0	0.0	0.0	0.0	0.0	+0.2	+2.2	+3.0	+0.8	0.0
T. Peckii	Gr.	4×	5×	2×	0	3×	2×	0	4×	3×	6×	4×	0
	P_H	7.0	4.8	6.2	8.6	5.6	6.6	2.0	6.0	3.4	3.6	5.2	5.4
	Diff.	0.0	+0.4	+0.8	0.0	−0.4	0.0	0.0	+1.2	+1.4	+2.2	+1.0	0.0
P. sapidus	Gr.	5×	1×	1×	1×	2×	2×	2×	4×	2×	4×	5×	4×
	P_H	7.0	5.0	6.4	8.6	5.2	6.6	7.0	6.6	3.6	3.4	3.4	6.0
	Diff.	0.0	+0.2	+0.6	0.0	0.0	0.0	0.0	+0.6	+0.8	+2.4	+2.8	−0.6
M. lacrymans	Gr.	1×	2×	0	0	2×	3×	4×	1×	4×	5×	6×	0
	P_H	7.0	4.6	2.0	8.6	5.6	6.6	7.0	7.0	2.8	2.8	3.0	5.4
	Diff.	0.0	+0.6	0.0	0.0	0.0	0.0	0.0	+0.2	+2.0	+3.0	+3.2	0.0
L. lepideus	Gr.	0	3×	0	0	1×	0	0	1×	2×	2×	2×	0
	P_H	7.0	4.8	6.8	8.6	5.2	6.6	7.0	7.0	3.2	4.0	5.6	5.4
	Diff.	0.0	+0.4	+0.2	0.0	0.0	0.0	0.0	+0.2	+1.6	+1.8	+0.6	0.0
D. confragosa	Gr.	1×	2×	1×	0	3×	1×	0	3×	1×	2×	3×	0
	P_H	6.8	5.0	6.8	8.4	5.6	6.6	7.0	7.6	3.0	2.8	3.4	5.4
	Diff.	+0.2	+0.2	+0.2	+0.2	−0.4	0.0	0.0	−0.4	+1.8	+3.0	+2.8	0.0
C. cerebella	Gr.	0	1×	0	0	1×	0	1×	0	3×	2×	2×	0
	P_H	7.0	5.2	7.2	8.6	5.4	6.6	7.4	7.4	2.8	3.2	3.4	5.4
	Diff.	0.0	0.0	−0.2	0.0	−0.2	0.0	−0.4	−0.2	+2.0	+2.6	+2.8	0.0
P. versicolor	Gr.	0	3×	5×	2×	5×	2×	1×	7×	3×	3×	4×	5×
	P_H	7.0	4.8	4.8	4.8	6.0	6.8	7.2	5.0	3.0	3.2	3.0	5.8
	Diff.	0.0	+0.4	+2.2	+3.8	−0.8	−0.2	−0.2	+2.2	+1.8	+2.6	+3.2	−0.4
P. lucidus	Gr.	3×	7×	2×	1×	2×	2×	0	4×	4×	5×	6×	6×
	P_H	5.0	4.2	6.6	8.6	5.6	6.6	7.0	4.2	4.2	4.4	5.0	5.2
	Diff.	+2.0	+1.0	+0.4	0.0	−0.4	0.0	0.0	+3.0	+0.6	+1.4	+1.2	+0.2
P. hirsutus	Gr.	0	2×	1×	0	3×	2×	0	4×	1×	2×	3×	5×
	P_H	7.0	4.2	4.4	8.6	5.6	6.6	7.0	4.8	3.2	3.2	3.2	5.2
	Diff.	0.0	+1.0	+2.6	0.0	−0.4	0.0	0.0	+2.4	+1.6	+2.6	+3.0	0.0
Control	P_H	7.0	5.2	7.0	8.6	5.2	6.6	7.0	7.2	4.8	5.8	6.2	5.4

* Gr. = growth; † P_H = H ion concentration; ‡ Diff. = change in H ion concentration due to growth.

There is a decrease in the amount of growth of *Daedalea quercina* and *Coniophora cerebella* when passing from the mono- to the tribasic potassium phosphate in Richards' solution. In *Merulius lacrymans, M. pinastri, Trametes Peckii, Pleurotus sapidus, Daedalea confragosa, Polystictus versicolor, P. hirsutus,* and *Polyporus lucidus* there is an increase, while in *Lenzites vialis* and *Lentinus lepideus* growth is equal in all cases.

In general, Dunham's solution and the pine decoction cannot be considered suitable media for these fungi, while maple sap compares favorably with the best solution (Richards' with the tribasic potassium phosphate).

In all of the fungi studied, except *Merulius pinastri,* the general tendency is to increase the active acidity during growth. However, there are exceptions to the general tendencies, indicating the fallacy of combining the results obtained from several fungi to draw sweeping conclusions as to a definite relation between the H ion concentration and growth of wood-destroying fungi as a group. Thus, we doubt the advisability of constructing a general curve with the average data derived from a limited number of fungi, as Meacham has done in a recent note.[1] This curve would not apply to many wood-destroying fungi, as the data in the table indicate.

The influence of the growth of the same fungi on the P_H value of different solutions is shown by a comparison of the data on Reed's and Richards' solutions. On Reed's solution containing the monobasic potassium phosphate all of the fungi made fair growth and raised slightly the P_H value; on the same solution with the dibasic potassium phosphate all of the fungi made slight growth only, and there is a tendency to raise the P_H value; while with the tribasic potassium phosphate those that grew exhibited a tendency to raise the P_H value. However, on Richards' solution with the monobasic potassium phosphate all of the fungi, with the exception of *Merulius pinastri,* lower even the very low P_H value of the solution; and

[1] Meacham, M. R. Note upon the hydrogen ion concentration necessary to inhibit the growth of four wood-destroying fungi. Science N. S. **48:** 499–500. *f. 1.* 1918.

although the P_H values of the solutions with the di- and tribasic potassium phosphates were originally greater than with the monobasic phosphate the lowering due to growth was very pronounced. This difference in the shifting of the active acidity due to growth in Reed's and Richards' solutions may be attributed to the fact that in Reed's solution the citrate radical of the sodium citrate acts as the source of carbon, thus liberating alkaline sodium compounds.

Conclusions

1. Many wood-destroying fungi are not suitable for growth experiments with liquid media.

2. With respect to the media employed and to the species studied, *Merulius pinastri, Polyporus lucidus, Polystictus versicolor, Pleurotus sapidus,* and *Trametes Peckii* grow best in the order named. Others grow well only on certain media, e. g., *Lenzites vialis, Daedalea quercina,* and *Merulius lacrymans* on Richards' solution.

3. Czapek's solution with the monobasic, and Richards' solution with the mono-, di-, and tribasic potassium phosphate proved generally to be suitable media. Thus, there is a decided indication of the desirability of selecting a specific medium for each fungus.

4. In the solutions studied the H ion concentration does not seem to be the limiting factor in growth, nor in general does it appear to be the factor (within the limits studied) which determines a desirable medium.

5. The shifting of the H ion concentration due to metabolism depends both upon the fungus and the medium.

6. No general statement can be made concerning the relation between the H ion concentrations of the culture media and the growth of wood-destroying fungi as a group.

MERULIUS IN NORTH AMERICA, SUPPLEMENTARY NOTES

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Merulius incarnatus Schw.—While recently privileged to study in the Farlow Herbarium, I found in the Curtis collection an authentic specimen of *Merulius incarnatus* Schw. from Schweinitz Herbarium in such excellent state of preservation that it corrects the error which has prevailed with regard to this species for nearly a century, and which I followed in my work on 'Merulius in North America."[1] *M. incarnatus* was published as dimidiate by Schweinitz[2] and also later by Fries[3] who had seen a specimen and emphasized the point by adding that this species is unique in its section in not being effuso-reflexed. The only dimidiate, white-spored *Merulius* which now occurs in the United States eastward from the Mississippi River is *Merulius rubellus* Peck, and this species does not range east of the Appalachian Mountains. As the descriptions of *M. incarnatus* by both Schweinitz and Fries apply better to *M. rubellus* than to the true *M. incarnatus,* there seemed no doubt that Schweinitz had collected as his type of *M. incarnatus,* in North Carolina in an isolated eastern station beyond its normal range, the fungus which has since become better known under the name *M. rubellus.*

The authentic specimen of *M. incarnatus* in Curtis Herbarium is not dimidiate, in spite of the statements by Schweinitz and Fries to the contrary, as already mentioned, but it is distinctly effuso-reflexed, with the upper surface of the broadly reflexed portion somewhat tomentose, pallid by age, and the hymenium ranging from flesh-pink to the red color of garnets, listed in Ridgway as garnet-brown and Hessian

1 Ann. Mo. Bot. Gard. 4 : 310. 1917.
2 Naturforsch. Ges. Leipzig Schrift. 1 : 92. 1822.
3 Elenchus Fung. 1 : 57. 1828; Epicrisis, 500. 1838.

brown. The hymenial folds form rather deep pores, radially elongated, transversely venose, and subdivided into smaller, angular pores. The fructification required an interval of several minutes after water was applied before its broad gelatinous layer softened sufficiently for sectioning. The spores are hyaline, allantoid, $3\times1\ \mu$.

This is the common species with snow-white effuso-reflexed pileus, garnet-colored hymenium, large decompound pores, and small allantoid spores, of the eastern United States. I have hitherto regarded it as a form of *M. tremellosus,* and have cited the various collections of it under *M. tremellosus* in my paper on 'Merulius.' Collections of it run down to *M. tremellosus* by the key to species of that paper, and I am not sure that it should not be referred here. However, upon examining critically seven European specimens of *M. tremellosus* which have been distributed in exsiccati, I find that none have the red hymenium of *M. incarnatus;* hence it may be practicable to separate *M. incarnatus* from *M. tremellosus* by the red hymenium of the former. Although I am unable to point out any other feature of difference, still such differences may be observed later.

M. incarnatus is common on stumps and logs of frondose species from Massachusetts to Texas, and in Missouri. June to December.

Specimens examined:

Exsiccati: Ravenel, Fungi Car. **2** : 22.

Massachusetts: Arlington Heights, *E. A. Burt;* Cambridge, *W. G. Farlow* (in Mo. Bot. Gard. Herb., 54920).

New York: East Galway, *E. A. Burt.*

Pennsylvania: *E. Michener, 478* (in Curtis Herb., and in Mo. Bot. Gard. Herb., 4077); Carbondale, *E. A. Burt;* Trexlertown, *W. Herbst.*

Virginia: Great Falls, *J. R. Weir, 8005* (in Mo. Bot. Gard. Herb., 54932).

North Carolina: *Schweinitz,* type (in Curtis Herb.).

South Carolina: *H. W. Ravenel,* in Ravenel, Fungi Car. **2** : 22; Santee, *H. W. Ravenel* (in Curtis Herb.); Society Hill (in Curtis Herb.).

Alabama: *Peters, 428* (in Curtis Herb.); Havana, *W. Trelease* (in Mo. Bot. Gard. Herb., 4071); Montgomery County, *R. P. Burke, 59, 114* (in Mo. Bot. Gard. Herb., 18206, 19801).
Texas: *Ravenel,* Dr. Billings, 114 (in Curtis Herb.).
Missouri: Creve Coeur, *E. A. Burt* (in Mo. Bot. Gard. Herb., 54328).

M. rubellus Peck.—This is a good species, although regarded by me formerly as a synonym of *M. incarnatus* Schw. in my work on 'Merulius in North America.' The description given there on p. 310 is correct for *M. rubellus,* as are also the distribution of the species and the citations of specimens examined. *M. rubellus* should be written in place of *M. incarnatus* in the key to species; and the citations of *M. incarnatus* in the synonymy and the last sentence of the comment on this species on p. 311 should be deleted.

M. hirsutus Burt.—Additional collections of this species which may be cited are:
Mexico: Jalapa, *W. A. & Edna L. Murrill, 65* (in N. Y. Bot. Gard. Herb., and in Mo. Bot. Gard. Herb., 55238); Oaxaca, Santa Ines del Monte, Zimatlan, 3000 ft. altitude, *C. Conzatti, 1377* (in Farlow Herb.).

M. lacrymans (Wulf.) Fr.—As this species is rare in North America, the following collections are recorded as additions to the list formerly published:
Massachusetts: Boston, on floor, tables, and walls up to a height of about 4 ft. in a laboratory of Massachusetts Institute of Technology, *W. G. Farlow* (in Farlow Herb.).
New Jersey: Newfield, on damp wall, *Lucian Fish,* from Ellis Herb. (in Farlow Herb.).

M. rimosus Berk.—In my work on 'Merulius in North America,' p. 327, I regarded this species as a probable synonym of *M. niveus* but had not been able to decide positively, because the cotype could not be found in N. Y. Bot. Gard. Herb. I have examined a portion of the cotype, communicated by Ellis, now in Farlow Herb., and it differs in no respect from *M. niveus.*

SEED DISINFECTION FOR PURE CULTURE WORK

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In recent years the growing of seed plants in pure cultures for both physiological and pathological purposes has become recognized as an important research method. When parasitic fungi are being studied in relation to their hosts it is necessary to control as many conditions as possible, and especially is it essential to prevent the entrance of other parasites. Moreover, when the relations of higher plants to organic nutrient solutions are being investigated, it is usually necessary to eliminate the possible action of all microörganisms. In order to obtain plants free from bacteria and fungi many and varied methods have been advocated. It is obviously necessary to begin with pure seed or with disinfected seed. This paper deals only with the latter possibility in the establishment of pure cultures.

An examination of the literature indicates that many germicides have been tried in an effort to disinfect seed. In most cases, the investigator has selected some particular germicide, which had proved favorable in one or more cases, and has attempted to make it applicable to all seeds under all conditions. Accordingly, when others attempt to use the method under different conditions poor results are obtained and the method may be considered a failure. There is much dissatisfaction, therefore, with any single germicide as an agent which will disinfect all seed under all conditions.

The chief observation that suggested a further test of disinfectants was the difficulty experienced, at the Missouri Botanical Garden, in obtaining consistent results in regard to seedlings relatively free from contamination for certain "immunity" experiments in which the author was associated with Dr. Duggar. It was found that seeds of the same varieties grown in different parts of the country had to be treated dif-

ferently and in some cases with a different germicide. This is perhaps only what is to be expected when one realizes that various soil bacteria and fungi show a wide range of tolerance towards the same toxic agent. A good example of this is cited by Makemson ('17) in his work with *Cladosporium fulvum*. He found that the spores of this fungus would germinate in a relatively strong solution of Bordeaux mixture in a hanging-drop culture. He noted further that under the conditions just mentioned the germ tubes grew as rapidly as in the control. On the other hand, the toxicity of Bordeaux mixture for most fungi is well known.

Many species of *Fusarium* are able to grow in a 1 per cent copper sulphate solution. Young and Cooper ('17), in their work on the determination of fungicidal coefficients, found that *Endothia parasitica* was killed at a dilution of 1 to 16,000 commercial lime-sulphur solution, while it required a 2 per cent solution of copper sulphate to kill this organism, both fungicides acting for a period of 15 minutes. On the other hand, when *Glomerella cingulata* was used and the same time interval employed, a dilution of 1 to 9600 commercial lime-sulphur was necessary to kill the spores, while a 1 per cent solution of copper sulphate was lethal. Lime-sulphur, therefore, is almost twice as toxic to the spores of *Endothia parasitica* as to those of *Glomerella cingulata*. The reverse is true in the case of copper sulphate. Horn ('04) found, in his work with some of the *Phycomycetes*, that exceedingly small traces of $CuSO_4$ would prevent growth. The success of the fungicide, then, must depend upon the species of bacteria or fungus present on the seed.

The nature of the seed will affect the selection of a germicide, since the resistance of different seed is as variable as that of spores. With respect to different germicides it is known that seeds may exhibit marked differences in permeability. The moisture content of the seed will also probably modify its resistance to any injurious agent. This fact has been frequently pointed out, and recently clearly demonstrated by Coons and McKinney ('17) in their work on the treatment

of grains for smuts, using formaldehyde as the disinfectant. They found that wheat seed high in moisture content absorbed the formaldehyde vapor very rapidly and were materially injured. Many other examples might be cited which, up to the present time, have seemed to indicate that no one germicide can disinfect under all conditions.

In his studies on the comparative viability of seed, fungi, and bacteria, de Zeeuw ('12) was unable by the usual method to free seeds of a certain amount of the disinfecting agent. He believed that the adherence of the agent permitted the continuance of antiseptic action, leading to a false sense of security at the time of making the transfer of the young seedling. He therefore devised a special apparatus by which it was possible to wash the seed thoroughly in order to free it from the germicide, and at the same time it prevented possible contamination from the outside during treatment.

The object of this work is to determine the effectiveness of some of the well-known disinfectants in relation to particular groups of seeds. No new methods have been devised nor new germicides used. In all cases the antiseptic action (that is, antiseptic action due to incomplete washing) of the disinfectant was prevented only when it hindered normal germination and growth of the seed. The percentage of germination and appearance of growth were the indicators in each experiment.

The first germicide employed in this work was commercial chloride of lime (largely calcium hypochlorite), as suggested by Wilson ('15), and his method was followed, namely, "Ten grams of commercial chloride of lime (titrating 28 per cent chlorine) is mixed with 140 cc. of water. The mixture is then allowed to settle for five or ten minutes and the supernatant liquid decanted off or filtered. The solution or filtrate, which contains about 2 per cent chlorine, is used as the disinfectant. The volume of the solution employed should be about five times or more the volume of the seed."

The seeds were placed in glass tumblers, in quantity not to exceed about one-fifth of the final volume. The tumblers were

then filled with the above-mentioned filtrate and covered with the top of a sterile Petri dish. The seeds were then removed at intervals of 2, 6, 11, and 20 hours. This was done with sterile forceps in the case of large seed and by means of a platinum spoon with small seed. In this case it was only necessary to drain the seed of the hypochlorite solution, as the antiseptic action did not hinder the germination and development of the seed. The transfer was made to small flasks or Petri dishes (depending on the size of the seed). In these flasks and Petri dishes there had been previously placed a small amount of a synthetic nutrient medium[1] in 1.5 per cent agar. Usually from 10 to 20 seeds were placed in each vessel. The flasks and Petri dishes were then placed in an incubator maintained at 25° C. The varieties of seed used in this experiment are given in table I. Not many kinds could be tried, but it will be noted that those experimented upon represent seeds of widely different families. The seeds were obtained from the Vaughan Seed Company, Chicago, Illinois, in all cases except where note is made to the contrary. More uniformity in results was obtained when seeds were secured from the same company. The results of the disinfectant are shown in table I.

According to the results indicated in the table, the commercial "chloride of lime" was very effective for sugar beet, cucumber, and lettuce, a higher percentage of germination and more vigorous seedlings being obtained by the use of this disinfectant. The most effective time intervals are shown in table IV. However, many seeds of other plants were obtained free from contamination, but the effectiveness of the germicide was not sufficiently high for practical purposes. Certain cucumber seed obtained from other seed companies could not be sterilized even with this disinfectant. Bacteria would continue to grow at the micropylar end of such of these seed as

[1] The nutrient medium was made up as follows:

Ammonium nitrate	1 gm.
Dihydrogen potassium phosphate	.5 gm.
Magnesium sulphate	.25 gm.
Iron chloride	Trace
Cane sugar	5 gms.
Distilled water	500 cc.

TABLE I

TREATMENT OF SEEDS WITH CALCIUM HYPOCHLORITE SOLUTION

Seed	Time of treatment (hrs.)	Germination (per cent)	Contamination after 48 hrs. (per cent)	Condition of seedling	Seed	Time of treatment (hrs.)	Germination (per cent)	Contamination after 48 hrs. (per cent)	Condition of seedling
Cucumber (*Cucumis sativus*)	2	95	20	Good	Cotton (*Gossypium*)*	6	85	100	Normal but seed covered with Fusarium
	6	95	15	Good		11	60	100	
	11	95	0	Very good		20	60	100	
	20	95	0	Injured		44	0	100	
Bean (*Phaseolus vulgaris* var. *nanus*)	2	45	20	Fair	Corn (*Zea Mays*)†	2	90	60	Good
	6	16	0	Poor		6	90	20	Good
	11	16	0	Poor		11	80	10	Good
	20	10	0	Poor		20	20	0	Poor
Radish (*Raphanus sativus*)	2	80	50	Growth inhibited	Alfalfa (*Medicago sativa*)	2	80	40	Good
	6	24	40			6	80	20	Good
	11	20	40			11	80	5	Good
	20	20	30			20	40	0	Growth inhibited
Lettuce (*Lactuca sativa*)	2	96	0	Good	Turnip (*Brassica Rapa*)	2	80	40	Good
	6	96	0	Good		6	80	10	Fair
	11	50	0	Poor		11	25	5	Growth inhibited
	20	0	0	Poor		20	0	0	Growth inhibited
Sunflower (*Helianthus annuus*)	2	90	100	Mouldy	Vetch (*Vicia sativa*)	2	90	20	Fair
	6	90	100			6	90	10	Growth inhibited
	11	90	100			11	40	0	
	20	60	100	Poor		20	0	0	
Sugar beet (*Beta vulgaris*)	2	40	10	Normal	Cucumber (*Cucumis sativus*)†	2	60	60	Normal
	6	40	0	Normal		6	60	30	
	11	40	0			11	40	10	
	20	25	0	Injured		20	20	5	

* Seed from Alabama. † Seed from St. Louis Seed Co.

remained viable after 20 hours' treatment with the hypochlorite solution. This, however, either indicates that disinfection did not occur or that the bacteria were shielded by being so far within as to be beyond the range of diffusion of the disinfectant. This disinfectant, then, is only effective with cer-

tain seeds, but by extensive tests many more might be added to the list.

Inasmuch as it is not possible to include a test which is in every sense a control, it may be well to state at this point that the germination of all seed was tested out under usual conditions for germination and in every case ranged from 95 to 100 per cent.

The second disinfectant employed was formaldehyde. It is especially toxic to many parasitic fungi inducing plant diseases. In practically all cases where it has been unsuccessfully used as a general germicide, the failure has been due rather to its injurious action upon the seed than to ineffectiveness in controlling fungi and bacteria. Seed injury has been especially noticeable where the gas method has been used. If the seed contains a large amount of water, the gas is absorbed more readily and the seed is promptly injured.

The method employed was the same as the previous one except that the seeds had to be washed free of the disinfectant. It was found after many trials that one thorough rinsing in sterile water was sufficient to remove enough of the formaldehyde to prevent the antiseptic action from affecting the germination and development of the seed. The time intervals for removing seed from the solution were usually 15, 30, and 45 minutes. The effectiveness of the fungicide is shown in table II. Formaldehyde was very effective for a large proportion of the seed used. In most cases, better results were obtained when the seeds were left in a stronger solution a shorter time. Excellent results were obtained when the seeds were first dipped momentarily in 70 per cent alcohol, or for a definite preliminary period in hydrogen peroxide. Where the seeds are fairly resistant this method of treatment is the logical one. Only fair results could be obtained with either cotton or potatoes. In the case of the former, the seed had to be delinted with concentrated H_2SO_4 and then subjected to the formaldehyde treatment. In the case of the potato it was difficult to prevent bacterial growth. Eyes with a very small portion of the potato (1 c. cm.) were washed for twelve hours,

TABLE II
TREATMENT OF SEEDS WITH FORMALDEHYDE

Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling	Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling
2 per cent solution					3 per cent solution				
Dwarf bean	15	96	20	Normal	Dwarf bean	15	96	40	Normal
	30	80	20	Normal		30	98	0	Good
	45	80	10	Normal		45	40	0	Fair
Cucumber	15	92	15	Normal	Cucumber	15	90	10	Normal
	30	80	5	Fair		30	96	0	Normal
	45	60	0	Poor		45	96	0	Poor
Lettuce	15	96	50	Fair	Cotton	15	60	80	Poor
	30	50	0	Poor		30	40	80	Poor
	45	10	0	Very poor		45	40	70	Poor
Radish	15	90	5	Good	Cotton¶	15	60	80	Normal
	30	45	0	Fair		30	60	80	Normal
	45	30	0	Very poor		45	50	5	Normal
	60	4	0	Very poor		60	40	0	Injured
Sugar beet	15	40	5	Very poor	Sunflower	15	90	80	Fair
	30	0	0	Very poor		30	96	70	Fair
	45	0	0	Very poor		45	70	40	Poor
Sunflower	15	85	50	Normal	Sunflower**	15	96	5	Good
	30	66	15	Normal		30	96	0	Good
	45	55	15	Normal		45	70	0	Growth inhibited
Wheat*	15	90	5	Very good	Wheat††	15	96	15	Good
	30	96	0	Very good		30	96	0	Good
	45	92	0	Very good		45	90	0	Fair
Barley	15	80	10	Fair	Alfalfa	15	90	20	Normal
	30	20	5	Poor		30	90	0	Normal
	45	0	0			45	60	0	Injured
Corn	15	90	20	Good	Corn	15	90	0	Normal
	30	90	10	Good		30	90	0	Slightly injured
	45	60	0	Fair		45	40	0	Injured
Garden pea	15	90	20	Normal	Potato	15	100	90	Normal
	30	80	0	Normal		30	100	90	Normal
	45	40	0	Injured		45	90	80	Normal
						60	90	80	Normal

* Preliminary treatment—soaked in water 12 hrs.
** Preliminary treatment—soaked in water 18 hrs.
¶ Preliminary treatment—soaked in water 18 hrs., heated to 68° C. for 15 min.
†† Preliminary treatment—soaked in water 6 hrs.

TABLE II (continued)

Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling	Seed	Time of treatment (minutes)	Germination (per cent)	Contamination (per cent)	Condition of seedling
4 per cent solution									
Cotton	15	60	80	Normal	Potato	15	100	90	Normal
	30	60	60	Normal		30	100	60	Normal
	45	40	20	Slightly injured		45	90	20	Normal
	60	20	20	Injured		60	40	10	Injured
Cotton†	15	60	40	Normal	Potato‡‡	15	100	90	Normal
	30	60	10	Normal		30	100	40	Normal
	45	40	0	Slightly injured		45	80	10	Normal
	60	20	0	Injured		60	40	10	Injured
Cotton‡	15	60	40	Normal	Potato||||	15	100	40	Normal
	30	50	10	Normal		30	100	10	Normal
	45	40	0	Slightly injured		45	60	0	Normal
	60	0	0	Injured		60	20	0	Slightly injured
Cotton||	15	60	10	Normal	Sunflower	15	20	0	Injured
	30	50	0	Normal		30	0	0	.....
	45	40	0	Slightly injured		45	0	0	.....
	60	0	0	Injured		60	0	0	.....
Potato 5% HCHO	15	100	50	Normal	Bean	15	90	0	Normal
	30	80	40	Normal		30	90	0	Injured
	45	60	20	Injured		45	20	0	Badly injured
	60	20	10	Injured		60	0	0	.....

† Preliminary treatment—soaked in 1% H_2O_2 for 10 min.
‡ Preliminary treatment—soaked in water 18 hrs., then heated to 65° C. for 15 min.
|| Lint removed with concentrated H_2SO_4.
‡‡ Preliminary treatment—cubes treated with 70% alcohol for 1 min.
|||| Preliminary treatment (see text).

then dipped momentarily in 70 per cent alcohol, then into a 1 per cent H_2O_2 solution for 10 minutes, and finally into the 4 or 5 per cent formaldehyde for 45 minutes. The percentage of germination was low, but after sprouts appeared growth was normal. In a few cases, sterile plants could not be obtained, and this was related to the source and kind of potatoes.

Alcohol was the next disinfectant tried. The same method as the foregoing was employed. The seeds were washed once to prevent further injurious action. No table of results is here included, as I was unable to free any seed from bacteria and still obtain fair germination. The same kinds of seeds as were used in table II were employed in this experiment. Weak alcoholic solutions (10–25 per cent) were used for long periods of time and stronger (25–70 per cent) for short periods as the seed would allow. In only a few cases were sterile seed obtained. Alcohol cannot be entirely discarded as a disinfectant, as it is very effective in a subsidiary way, as when seeds are first immersed in strong alcohol and then followed by another disinfectant. It serves to remove air cavities which would not otherwise allow proper contact for the disinfectant.

The next disinfectant used was mercuric chloride. This has also been used very extensively for seed treatment. It is effective in dilute solutions, and for this reason it is necessary to remove practically all the solution from the seed, as its antiseptic action prevents the germination and growth of the seed. Some seeds are very easily injured by dilutions that will not kill certain fungi or bacteria. This is especially true with barley. It is also effectively used as the chief disinfectant when preceded by hydrogen peroxide or alcohol. The method used was the same as given with the previous disinfectants. The seeds were washed thoroughly of the $HgCl_2$, as its antiseptic action was found to be injurious in most cases. I was able to disinfect many seeds with mercuric chloride that could not be freed from contamination by any other disinfectant. Table III shows the results with this disinfectant.

It might be stated again that the seeds used in this table could not in every case be made free from contamination, but when good viable seed were used, a high percentage of the trials proved successful. Many hundred plantlets were obtained from seed disinfected by the above methods. From the data at hand it appears that a previous treatment with a good bactericide, such as alcohol or hydrogen peroxide, is to be desired. There are many seeds that will withstand a suf-

TABLE III

TREATMENT OF SEEDS WITH MERCURIC CHLORIDE

Seed	Time of exposure (min.)	Germination (per cent)	Contamination (per cent)	Condition of seedling
.1 per cent solution				
Corn	1	90	40	Normal
	10	90	20	Normal
	20	80	0	Normal
	40	40	0	Injured
Field peas	1	96	30	Normal
	10	96	10	Normal
	20	96	0	Normal
	40	60	0	Injured
Wheat	1	90	35	Normal
	10	10	10	Normal
	20	20	5	Injured
	40	0	0	Injured
Dwarf bean	1	96	60	Normal
	10	96	40	Normal
	20	60	10	Injured
	40	20	5	Injured
Radish	1	90	60	Normal
	10	90	50	Normal
	20	90	20	Normal
	40	60	0	Normal
Alfalfa	1	86	70	Normal
	10	78	60	Normal
	20	70	10	Normal
	40	60	0	Normal
Garden peas	1	96	75	Normal
	10	96	70	Normal
	20	96	40	Normal
	40	90	20	Normal
Barley	1	60	75	Normal
	10	60	5	Normal
	20	40	0	Injured
	40	0	0	
.5 per cent solution				
Garden peas	1	96	40	Normal
	10	96	20	Normal
	20	90	0	Normal
	40	80	0	Slightly injured
Dwarf bean	1	94	40	Normal
	10	94	10	Normal
	20	70	0	Normal
	40	30	0	Injured
Garden cress (*Lepidium sativum*)	1	90	40	Normal
	10	90	20	Normal
	20	80	0	Normal
	40	30	0	Injured
Morning glory (*Ipomoea purpurea*)	1	96	20	Normal
	10	96	10	Normal
	20	90	0	Normal
	40	60	0	Injured
Nasturtium (dwarf)	1	90	60	Normal
	10	90	40	Normal
	20	90	0	Normal
	40	90	0	Injured
Onion*	1	80	90	Normal
	10	80	40	Normal
	20	80	0	Normal
	40	60	0	Injured
Lupinus albus†	1	90	80	Normal
	10	90	80	Normal
	20	90	60	Normal
	40	80	10	Normal
Lupinus albus‡	1	90	60	Normal
	10	90	50	Normal
	20	90	10	Normal
	40	75	0	Normal

* Preliminary treatment—seed soaked in H_2O 12 hrs., then 1% H_2O_2 for 1 min.
† Preliminary treatment—soaked in 70% alcohol for 1 min.
‡ Preliminary treatment—soaked in H_2O_2 for 10 min.

ficiently long treatment of both agents mentioned, and some, such as barley, that are very sensitive and cannot be so treated. I have not been able to free barley from contamination in more than 75 per cent of the seed used. There are many other seeds that are easily killed by any germicide that will be effective against the adhering microörganisms. In conclusion, therefore, it would seem necessary to continue to treat the various seed differently and with different disinfectants. One of the most important considerations is that there are undoubtedly different contaminating organisms.

TABLE IV

SUMMARY GIVING BEST TREATMENTS FOR VARIOUS KINDS OF SEEDS

(For percentage of germination in each case, see Tables I, II, and III)

Disinfectant	Seed	Strength of disinfectant	Time of exposure	Previous treatment
Calcium hypochlorite ($CaOCl_2$)	Cucumber	Full	11 hrs.	None
	Lettuce	Full	2–6 hrs.	None
	Sugar beet	Full	11 hrs.	70% alcohol, 1 min.
Formaldehyde (HCHO)	Bean	2–3%	30 min.	None
	Cotton	3–4%	45 min.	Soaked in H_2O, 12 hrs., lint removed with H_2SO_4
	Corn	2–3%	15 min.	None
	Cucumber	2–3%	30 min.	None
	Alfalfa	3%	30 min.	None
	Sunflower	2–3%	30 min.	Soaked in H_2O, 18 hrs.
	Wheat	2–3%	30 min.	Soaked in H_2O, 6 hrs., 1% H_2O_2, 10 min.
	Potato	3–4%	45 min.	70% alcohol, 1 min.
Mercuric chloride ($HgCl_2$)	Corn	.1%	20 min.	None
	Field peas	.1%	20 min.	None
	Radish	.1%	40 min.	None
	Alfalfa	.1%	40 min.	None
	Barley	.1%	10 min.	70% alcohol momentarily
	Garden peas	.1%	20 min.	None
	Dwarf bean	.5%	20 min.	None
	Garden cress	.5%	20 min.	None
	Nasturtium	.5%	20 min.	Soaked in H_2O, 12 hrs.
	Onion	.5%	20 min.	Soaked in H_2O, 12 hrs., then in 1% H_2O_2, 10 min.
	Lupines	.5%	40 min.	1% H_2O_2, 10 min.; 70% alcohol, 1 min.

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SEED DISINFECTION FOR PURE CULTURE WORK: THE USE OF HYPOCHLORITES

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INTRODUCTORY

For a long time it has been clear that much light may be thrown on many fundamental problems in seed plant nutrition as well as in physiological pathology through the use of pure cultures of certain seed plants. In those cases where the seed are produced in pods, solid fruits, or within other thoroughly protective coverings, it is, as a rule, a relatively simple matter to secure seed in season entirely free from contamination. It is only necessary to employ the usual bacteriological precautions, opening the maturing pods or fruits with care and removing the seed to sterile containers, in which they may be kept until required. Beans, peas, radishes, tobacco, tomatoes, and various cucurbits or melons are among those plants easily handled in this way. The difficulty, however, even with these seed, lies in anticipating what may be needed out of season. With the majority of seed, moreover, it would not be practicable to use the isolation method either because of structural difficulties or of inaccessibility of fruiting plants.

The experiments of Wilson[1] on the use of commercial chloride of lime (in part calcium hypochlorite) have been the first definite application of the value of this well-known disinfecting agent to plant physiological study with seed plants. In the disinfection and antiseptic treatment of wounds, extensive studies have been made during this war with the use of hypochlorous acid, the hypochlorites, and related compounds, as a result especially of the investigations of Dakin[2]

[1] Wilson, J. K. Calcium hypochlorite as a seed sterilizer. Am. Jour. Bot. 2 : 420–427. 1915.

[2] Dakin, H. D., and E. K. Dunham. A handbook on antiseptics. 126 pp. New York, 1917.

and his associates. In general, this work has emphasized the value of a series of compounds containing "active chlorine," by which term Dakin infers a connotation of "the ability of any particular substance to part with chlorine, free or combined, in such a way that it can effect the chlorination of bacterial and other proteins." Through the NH groups of their constituent amino-acids the proteins are subject to attack by such chlorinated agents, whereby in the first step the Cl is substituted for the H-atom in the group mentioned with the formation of chloramines.

Materials and Methods

In the preliminary work here reported we have not departed from the readily obtainable commercial products, namely, (1) commercial chloride of lime, or "bleaching powder," (2) "chlorinated potassa" (a liquid product recognized by certain St. Louis manufacturers as "eau de Javel"), (3) solid sodium hypochlorite, and (4) Dakin's soluble chloramine T. These we have compared with a few standard disinfectants of other groups. The commercial products vary somewhat in composition, but these differences, in our experience, are not so great as to interfere with this type of practical work. A careful study of standardized preparations is, however, planned. Chloramine T, or chlorazene, is the abbreviated or trade name for sodium-toluene-para-sulphochloramide. No experimental work was done with the other Dakin products of this class.

The chlorinated lime, designated 10 per cent, was prepared in the following way: Ten grams of a standard commercial product were stirred into 100 cc. distilled water. After standing 10 minutes the supernatant liquid was filtered and the filtrate employed. Other concentrations were prepared in an analogous manner. The commercial Javel water was used as if it were a pure substance, 10 and 20 per cent solutions referring respectively to the use of 10 and 20 cc. of the commercial product with enough water in each case to make 100 cc. All necessary precautions have been taken to prevent accidental contamination. The technique of handling treated seed

has been invariably carried out in a transfer room repeatedly steamed to insure the precipitation and fixation of dust particles. In some cases the seed were preliminarily immersed in running water for from 4 to 16 hours, in other cases dry seed were treated directly.

In the earlier work the selected seed were placed in small cheese-cloth bags, and these immersed in covered vessels containing the disinfecting solutions employed. After the interval of treatment the bags were transferred to jars of sterile distilled water for from 15 minutes to 1 hour, and when it seemed desirable a second quick rinsing was given. The contents of each bag was then carefully dumped into a sterile Petri dish. In later work the washed or soaked seed were carefully placed in sterile Erlenmeyer flasks, and the disinfectant then poured in, a separate flask being used for each lot of seed to be treated for any interval of time as well as by each concentration of disinfecting solution. After treatment the seed were twice shaken with sterile water, the second wash water remaining not less than 15 minutes. The seed were then transferred to a Petri dish by means of a metal spoon. All implements employed were sterilized by dipping in alcohol and then promptly burning this off.

The purity and germination of the seed were then followed after their transfer to large Petri dishes containing standard potato decoction agar. Care was taken to insure intimate contact of the seed with the medium. From 15 to 50 seed, depending upon size, were usually arranged in each dish, and each test duplicated.

Inasmuch as the essential thing in such work is to obtain a high percentage of germinating seed free from contamination and readily transferable to other cultures, the above methods have seemed entirely adequate, and complicated apparatus, such as that devised by de Zeeuw[1], is not merely unnecessary, but it is in general impracticable.

In the true sense of the word "control" experiments are not possible in this work, and no attempt is made to include ex-

1 Zeeuw, R. de. The comparative viability of seeds, fungi and bacteria when subjected to various chemical agents. Centralbl. f. Bakt. II. 31 : 4–23. *1 f.* 1911.

periments thus designated. All seed not disinfected will exhibit contamination on agar. On the other hand, elaborate experiments might have been made to determine the effect of the disinfectant upon the seed, and in a certain sense these would serve as controls. Our object, however, has been simply to use seed which under the usual conditions of the germinator exhibit a high percentage of germination, so we have merely assured ourselves of the capacity of the seed employed to germinate satisfactorily. It is admitted that germination in a Petri dish on agar is not comparable to germination in a germinator.

Experimental Data and Discussion

At the outset it should be definitely acknowledged that as a general principle the practically perfect disinfection of seed by chemical agents is only possible when the contaminating organisms of the seed are superficial, or largely superficial. This conclusion is drawn from a variety of observations and experiments, the general result of which is too obvious to require elaborate data and discussion but merits mention in respect to some pronounced instances. Experiments have been made with seed suspected of more than superficial contamination in the case of corn, sunflower, squash, tomato, and *Melilotus*. In the first case, corn of the 1917 crop was obtained in which a discoloration of the micropylar end of the seed was characteristic. The normal maturity of this crop in the Middle West was more or less affected by early frost, and in some sections subsequent wet weather induced visible mouldiness. The seed used exhibited no macroscopically visible infection of the cob, but such infection was inferred. After treatment with various disinfecting agents the majority of these seed and any contaminating organisms were either killed by the agent, or, in the agar cultures, there was a growth of fungous hyphae from the discolored micropylar end. Such seed were necessarily discarded. The other seed mentioned had been stored under moist conditions, favoring the development of moulds and bacteria, and in no case was it possible to disinfect any reasonable percentage without injury in respect to

the capacity for vigorous germination. Similar observations upon the seed of certain grasses, sorghums, etc., which had undergone considerable heating during the curing process have led to the conviction that penetration of the seed by microörganisms, especially fungi, is not infrequent, and therefore a certain "purity" of the seed employed is requisite.

In table I are shown data obtained from the treatment of Canada field peas and corn with chlorinated lime, chlorinated potash, and chlorazene. In this case the first-mentioned disinfectant was more injurious to peas than in any other test made. The results with chlorinated potash were considered particularly good for the preliminary trials, while the results with chlorazene were disappointing.

Before proceeding further, tests were made, for comparison, with formalin and mercuric bichloride, and a second trial of chlorazene was included, as given in table II. The use of mercuric bichloride with such seed gave perfect disinfection, but these, as well as subsequent experiments, seemed to indicate that whenever this result was accomplished the injury to the seed was considerable. Various grades of alcohol, from 20 to 95 per cent, were also used in this series, the treatment

TABLE I

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 16 HOURS) BY CHLORINATED LIME, CHLORINATED POTASH (JAVEL WATER), AND CHLORAZENE. FINAL OBSERVATIONS AFTER 72 HOURS, ROOM TEMPERATURE

	Disinfectant	Treatment 3½ hours		Treatment ½ hour	
		% Germination	% Contamination	% Germination	% Contamination
Peas	25% chlor. lime	12	0	25	0
	15% chlor. lime	6	0	...	....
	25% chlor. potash	88	0	100	0
	10% chlor. potash	94	0	88	0
	4% chlorazene	0	4	0	0
	2% chlorazene	6	3–4	25	0
	1% chlorazene	42	0	87	0
Corn	25% chlor. lime	80	0	87	0
	15% chlor. lime	75	7	80	7
	25% chlor. potash	88	0	100	3
	10% chlor. potash	94	3–4	100	0
	4% chlorazene	94	0	100	11
	2% chlorazene	88	7	66	10
	1% chlorazene	75	0	87	7

TABLE II

DISINFECTION OF CANADA FIELD PEAS BY FORMALIN, MERCURIC BICHLORIDE, AND CHLORAZENE. FINAL OBSERVATIONS AFTER 96 HOURS, 26° C.

Disinfectant	Treatment 2 hours			Treatment 1 hour		
	% Germination	% Contamination	Condition	% Germination	% Contamination	Condition
* .2% formalin	77	50	Fair	93	10	Good
.2% formalin	70	66	Poor	83	43	Good
* .1% $HgCl_2$	16	0	Injured	24	0	Injured
.1% $HgCl_2$	47	0	Fair	76	0	Fair
*4% chlorazene	0	10	Injured	0	50	Fair
4% chlorazene	16	40	Fair	56	13	Fair
*2% chlorazene	33	6	Fair	43	6	Good
2% chlorazene	40	33	Good	63	6	Good
*1% chlorazene	86	20	Good	80	10	Good
1% chlorazene	83	30	Good	80	30	Good

* In these cases the seed were soaked for 16 hours prior to treatment, while in the other cases dry seed were treated.

being 5, 20, and 60 minutes. After 60 hours practically every seed was contaminated, so that the results are not tabulated.

In table III are given further results with chlorinated lime and potash. The intervals employed are too short for best results, so that the percentage of contamination runs high. Moreover, these experiments were made several months later than those included in tables I–II, and the seed were not so fresh. It seems clear, however, that short treatments are not satisfactory, and the value of longer intervals is particularly emphasized later.

A further extensive test of chlorinated lime, employing intervals of treatment up to 3 hours and using the freshest seed available, was made with the special view of determining the effect of the agent on the germination of the seed. The results are given in detail in table IV. The effectiveness of this disinfectant with corn and cucumber is clear. The percentage of germination with peas and radish is relatively low. This lot of radish seed proved difficult to sterilize, and the presence of a resistant organism spreading rapidly over the surface of the dishes tended to reduce the percentage of germination.

The test of sodium hypochlorite was made by preparing the concentrations indicated in table V from the solid substance.

TABLE III

DISINFECTION OF SEED BY CHLORINATED LIME AND CHLORINATED POTASH. FINAL OBSERVATIONS AFTER 96 HOURS

	Disinfectant	Treatment 1 hour			Treatment ½ hour		
		% Germination	% Contamination	Condition	% Germination	% Contamination	Condition
Peas	*20% chlor. lime	34	3	Fair	23	17	Fair
	20% chlor. lime	100	6	Good	87	0	Good
	*10% chlor. lime	37	13	Good	77	3	Fair
	10% chlor. lime	93	13	Good	93	0	Good
	* 5% chlor. lime	75	6	Fair	77	General	Fair
	5% chlor. lime	75	0	Good	100	0	Good
	*20% chlor. potash	80	20	.....	80	6	.....
	20% chlor. potash	75	13	.....	87	6	Good
	*10% chlor. potash	77	22	Good	77	50	Good
	10% chlor. potash	87	6	Good	93	6	Good
	* 5% chlor. potash	94	16	Good	77	50	Good
	5% chlor. potash	93	13	Good	87	0	Good
Corn	*20% chlor. lime	83	0	Fair	89	0	Good
	20% chlor. lime	88	20	Good	67	0	Good
	*10% chlor. lime	97	6	Good	67	0	Fair
	10% chlor. lime	95	6	Good	72	0	Fair
	* 5% chlor. lime	88	6	Good	78	10	Fair
	5% chlor. lime	88	0	Good	88	0	Fair
	*20% chlor. potash	75	3	Good	75	0	.....
	20% chlor. potash	67	6	Good	77	0	.....
	*10% chlor. potash	86	0	Good	67	General	Fair
	10% chlor. potash	83	13	Good	83	0	Fair
	* 5% chlor. potash	81	23	Good	75	General	Fair
	5% chlor. potash	83	6	Good	77	General	Fair

* In these cases the seed were soaked for four hours prior to treatment, while in the other cases dry seed were treated.

Although rather more erratic than results with other hypochlorites, the trial was of importance inasmuch as some dishes were free, or practically free, of contamination, and the percentage of germination relatively high. After further tests, however, it appeared that stronger solutions might be employed, and later a series of experiments was made in which this agent was compared with the potassium salt and with chlorazene, as shown by the data in table VI. These results are much more favorable than before for sodium hypochlorite; but the uniformly high percentage of germination, the good condition of the seedlings, and the freedom from contamina-

TABLE IV

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS) BY CHLORINATED LIME. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	2	72	0
	10	1	90	0
	10	½	90	0
	5	3	72	2
	5	2	84	0
	5	1	86	6
Peas	10	2	12	0
	10	1	24	0
	10	½	18	4
	5	3	38	0
	5	2	24	6
	5	1	32	4
Cucumber	10	2	84	0
	10	1	84	0
	10	½	68	2
	5	3	64	0
	5	2	82	8
	5	1	84	88
Radish	10	2	12	18
	10	1	30	14
	10	½	38	14
	5	3	4	56
	5	2	8	58
	5	1	28	24

tion (not perfect in the case of radish) where the potassium salt was employed, mark this as far more reliable under these conditions.

During the progress of this work other investigations were in progress by one of us and by graduate students, in which germinating seed free of contaminating organisms were required. The Javel treatment was employed, and with the consent of those who coöperated in this work, Dr. W. W. Bonns and Mr. T. Matsumoto, we are enabled to report the following facts: Of 500 seed of Canada field peas treated 3 hours with a 10 per cent solution the germination was 90 per cent and the contamination less than 1 per cent. In treating tobacco seed, 2- and 3-hour intervals proved inadequate; but a 4-hour interval with 12 per cent Javel water gave a contamination of less

TABLE V

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS AT 23° C.) BY SODIUM HYPOCHLORITE. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	2	96	2
	10	1	86	30
	10	½	92	4
	5	3	90	0
	5	2	92	0
	5	1	90	44
Peas	10	2	26	35
	10	1	30	96
	10	½	58	52
	5	3	40	58
	5	2	28	30
	5	1	30	20
Cucumber	10	2	88	50
	10	1	96	72
	10	½	92	100
	5	3	92	30
	5	2	82	14
	5	1	94	98
Radish	10	2	26	58
	10	1	18	10
	10	½	46	14
	5	3	46	6
	5	2	24	2
	5	1	26	4

than 2 per cent when 12 plates of about 30 seed each were sown. At the same time small quantities (2 dishes each) of lettuce, navy beans, and lima beans were similarly treated, with no contamination in any dish.

It was suggested that discontinuous disinfection might prove serviceable and practicable in work of this type, just as discontinuous sterilization by heat is so effective. Accordingly, experiments were arranged in which the dry seed were treated for 2 hours in deep Petri dishes with 20 per cent Javel water, then, after pouring this off, the seed were rinsed, and finally left in an incubator at room temperature for 2 days. The same treatment with rinsing was then repeated, and the seed placed for germination as in other cases. The results are shown in table VII, and these indicate complete disinfec-

TABLE VI

DISINFECTION OF SEED (AFTER IMMERSION IN WATER FOR 4 HOURS AT 23° C.) BY CHLORAZENE, CHLORINATED POTASH, AND SODIUM HYPOCHLORITE. FINAL OBSERVATIONS AFTER 96 HOURS

Disinfectant	Kind of seed	% Germination	% Contamination	Condition
1% chlorazene	Corn	100	50	Good
	Peas	26	100	Good
	Cucumber	100	...	Injured
	Radish	40	100	Good
20% chlor. potash	Corn	96	0	Good
	Peas	94	0	Good
	Cucumber	84	0	Good
	Radish	64	16	Good
20% sodium-hypochlorite	Corn	100	2	Good
	Peas	22	50	Injured
	Cucumber	96	0	Good
	Radish	12	0	Good

tion, as well as an unusually high percentage germination as compared with the usual percentage when the same seed are treated with any such disinfecting agent and placed directly on agar. The principle involved may possibly be widely applicable and deserves consideration in other work.

The results of similar tests with chlorinated lime, varying, however, the time intervals and the concentration of the disinfectant, are given in table VIII.

In addition to the results reported in full, extensive experiments were made with sodium chlorate and calcium sulphite. Concentrations of 10 and 5 per cent of the pure reagents were employed for various intervals, but in all cases 100 per cent

TABLE VII

DISINFECTION OF SEED BY 20 PER CENT CHLORINATED POTASH,—DISCONTINUOUS TREATMENT. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Germination	% Contamination	Condition
Corn	100	0	Fine
Peas	90	0	Good
Cucumber	100	0	Fine
Radish	50	20	Fair

TABLE VIII

DISINFECTION OF SEED BY CHLORINATED LIME,—DISCONTINUOUS TREATMENT. FINAL OBSERVATIONS AFTER 96 HOURS

Kind of seed	% Concentration	Interval of treatment, hours	% Germination	% Contamination
Corn	10	1	72	0
	*10	1	60	0
	*10	½	100	0
	5	2	56	28
	*5	2	100	0
	*5	1	96	0
Peas	10	1	20	8
	*10	1	52	0
	*10	½	24	50
	5	2	16	100
	*5	2	60	0
	*5	1	40	4
Cucumber	10	1	92	0
	*10	1	36	0
	*10	½	80	0
	5	2	60	100
	*5	2	72	12
	*5	1	100	0
Radish	10	1	0	12
	*10	1	8	16
	*10	½	14	100
	5	2	0	48
	*5	2	0	56
	*5	1	18	...

* In these cases the seed were soaked for 4 hours prior to treatment, while in the other cases dry seed were treated.

of contamination occurred, so that these and related substances were believed to be unsatisfactory for the purpose of this study.

Particular stress has been laid by de Zeeuw on the difficulty of properly washing off the disinfectant. He argues that quite commonly there may be transferred with the seed a sufficient amount of the disinfectant to insure antiseptic action during the germination of the seed on agar, but the unkilled germs may develop later—when the seed are transferred to final cultures. With the hypochlorites we have not been able to detect any such possibility, even when intentionally making the washing process less thorough than usual. Seed of beans,

corn, and peas, merely dipped in water—after disinfection—and then once in a dilute suspension of *Bacillus mycoides* before being placed on agar for germination, showed 100 per cent of contamination after repeated trials. Possible difficulties with the salts of heavy metals were not tested.

Definite experiments are under way to test the value of certain commercial hypochlorites in the disinfection of seed for agricultural purposes.

Summary Recommendation

As a practical result of the experiments on seed treatment reported it is believed that, taking into consideration both (1) the capacity of the seed for germination after treatment and (2) the relative freedom from contamination by microörganisms, the commercial "chlorinated potassa," or Javel water, is the most satisfactory agent which has yet been considered. The length of time required for practically perfect disinfection of certain seed in our experiments, with concentrations of 10–20 per cent, is 3 hours or longer. In some cases interrupted disinfection fully warrants the extra trouble and delay. The value of soaking the seed previous to the longer interval treatments is doubtful. Nevertheless, a preliminary thorough washing of all seed, with removal of decayed and imperfect ones, is advisable. A study of standardized hypochlorites is essential.

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AN EDIBLE GARDEN HEBELOMA[1]

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The species of *Hebeloma* have been so invariably found in woods in autumn in the past that it occasioned great surprise to find a fine species of this genus in great abundance, June 3, in cultivated borders of the Missouri Botanical Garden. The mycelium of this species was well developed and could be followed to the strawy manure which had been spaded underground in the borders.

Species of *Hebeloma* have usually a nauseous odor or taste, or an odor or taste of radishes, or may be bitter, and are regarded as unwholesome and, in some cases, even poisonous. Specimens of this garden collection were of good size, with pileus 4–10 cm. in diameter, not infested with larvae, not rapidly putrescent, with a pleasant farinaceous taste and odor. This species gave promise of being a very desirable acquisition if its edibility could be established. This was done by cooking specimens in butter and eating a small portion of a pileus with other food at dinner. As no disagreeable symptoms were experienced over night, double the quantity of the fungus was eaten at breakfast. This procedure was carried on until three fructifications were eaten at one time, when others also ate the fungus with equal freedom and decided that this species is palatable and has a delicious characteristic flavor.

The specimens did not have as large size and as great weight

[1] Issued October 11, 1919.

as those of *Agaricus campestris* when the latter is grown as a mushroom for the market but they are of good size and weight, are firm and keep well, and have a flavor of good quality which is distinct from that of *Agaricus campestris*. These are desirable qualities in a market species of mushroom. The origin of the mycelium in the strawy manure which was spaded into the soil suggests that preparation of the spawn and method of growing under cultivation might be the same as those employed for the common mushroom, *A. campestris*.

This garden *Hebeloma* is apparently of local occurrence, for its characteristics do not agree with those of any species heretofore known. It may be that the normal season of this mushroom is spring or early summer, as is the case with *Pholiota vermiflua*, a species which was abundant in the Missouri Botanical Garden at the same time. The month of May preceding had been very wet and with frequent storms throughout the month. The name and description of this species are as follows:

Hebeloma hortense Burt, n. sp. Plate 3.

§*Denudata*. Pileus glabrous, veil absent from the first.

Type: in Mo. Bot. Gard. Herb., 54130, and in Burt Herb.

Pileus fleshy, convex, umbonate, becoming expanded, even, glabrous, pale Isabella-color when moist, becoming cartridge-buff, with the umbo pinkish buff, the margin slightly inrolled when very young; flesh whitish, farinaceous; odor not disagreeable, not of radishes; lamellae broad, slightly sinuate, moderately close, white at first, becoming Isabella-color, the edge entire and not distilling drops; stem fleshy, hollow, equal or slightly bulbous at the base, fibrillose, mealy above when young, with no trace of a veil or cortina; spores Rood's brown in spore collection, even, 10–11 x 6 μ, borne in fours on protruding basidia; no cystidia present or not noteworthy.

Pileus 4–10 cm. broad; stem 4–9 cm. long, 4–10 mm. thick.

In cultivated borders where a straw manure had been worked into the ground. St. Louis, Missouri. Abundant in early June after prolonged rains.

The fructifications occur singly or in small clusters of two or three. This species is noteworthy in its genus by absence of

viscidity and odor of radishes, and by its large size and occurrence in abundance in cultivated ground. Nearly all other species of *Hebeloma* are inhabitants of forests and occur there sparingly. Fresh specimens have a pleasant farinaceous taste and odor, and keep well. This species is edible and with a pleasant and distinctive flavor.

Explanation of Plate

PLATE 3

The figures of this plate have been reproduced natural size.

A cluster of two specimens viewed from above to show form of pileus.

A single specimen showing the lamellae and stem.

Median vertical section through pileus and upper part of stem to show breadth and attachment of the lamellae.

Transverse section of the hollow stem.

A very young fructification split lengthwise to show slightly inrolled margin of pileus and the absence of a veil or cortina.

BURT—HEBELOMA HORTENSE

PROTOMERULIUS FARLOWII BURT, N. SP.[1]

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During his last illness and only two days before his death, Dr. Farlow had mailed to me a very interesting fungus which he collected near his summer home at Chocorua, New Hampshire. This fungus, which I have named *Protomerulius Farlowii*, has apparently more minute pores than have been recorded for any species heretofore described. The pores are so minute that to the naked eye the fructification has the aspect of a very thin *Sebacina* with its hymenial surface slightly pruinose. This pruinose surface becomes barely visible as irregularly angular and somewhat sinuous pores with very thin dissepiments when viewed through a pocket magnifier of fine definition having a magnification of 10 or more diameters, and is beautifully shown under the compound microscope with an objective of about 16 mm. focal distance.

Under this higher magnification the dissepiments appear as thin, irregular folds up to 30 μ high and about 20 μ thick, with the edge acute and lacerate. The angular pores are incompletely enclosed by the dissepiments; the hymenial configuration is that of *Merulius* rather than *Poria*.

Preparations of the hymenium show longitudinally cruciately septate basidia 9–10$\times$7 μ. Hence this fungus is a member of the *Tremellaceae* and has the hymenial configuration of a *Merulius*.

A. Möller collected at Blumenau, Brazil, a fungus having the form of a *Merulius* and longitudinally cruciately septate basidia, which he published[2] as *Protomerulius brasiliensis* new genus and species. Although the hymenial folds and pores are much smaller and less perfectly developed than those of *Protomerulius brasiliensis*, the generic description of *Protomerulius* applies well to the New Hampshire specimen.

Although 24 years have elapsed since the publication of *Proto-*

[1] Issued October 11, 1919.

[2] Bot. Mitt. a. d. Tropfen 7:60. 1895; 8:129, 172. *pl. 3. f. 3, 4, pl. 5. f. 36.*

merulius I fail to find record that collections referable to this genus or its single species have been made elsewhere in this rather long interval of active mycological exploration. It is therefore remarkable that the presumably tropical genus *Protomerulius* should have so noteworthy a species as *P. Farlowii* in northern New Hampshire at a rather high altitude.

The color of the specimens of *P. Farlowii* is noted as purple when in vegetative condition and suggestive in aspect of a species of *Tulasnella*, but this color was soon lost in drying and the specimens are now pale olive-gray of Ridgway. The fructifications occur on the surface of decayed coniferous wood, on the rough surface of which a slender foliaceous hepatic is present also.

Vertical sections through the fructification and substratum show the fructification to be a continuous compact membrane 10–15 μ thick; this membrane is composed of longitudinally arranged, thin-walled, hyaline hyphae crowded closely together. Branches from the hyphae of this membrane curve outward here and there and terminate in clusters of basidia. The basidia are somewhat interruptedly arranged in the hymenium rather than densely. At intervals of about 40 μ hyphae grow outward from the membrane to form the tramal tissue of the folds or dissepiments. These folds are about 30 μ high and 20 μ thick and covered by the hymenium. The membranous layer of the fructification is elevated about 40 μ above the surface of the wood and supported by groups of hyphae which arise from the substratum. These details are shown in the accompanying text-figures.

The formal description of this species is as follows:—

Protomerulius Farlowii Burt, n. sp.

Type: in Farlow Herb. and Mo. Bot. Gard. Herb.

Fructifications resupinate, effused, gelatinous, membranaceous, very thin and tender, separable with care when moist, "purple" when fresh, becoming pale olive-gray upon drying, pruinose to the naked eye, but showing under the microscope an imperfectly porose surface with thin, irregular folds and dissepiments more or less lacerate, the edges thin; pores angular-sinuose, about 40 μ in diameter or 25 to a mm.; in structure 20–30 μ thick, with a compact subhymenial layer 10–15 μ thick,

composed of densely and longitudinally arranged, hyaline, thin-walled hyphae 3 μ in diameter; subhymenial layer elevated above the substratum by scattered clusters of hyphae; basidia longitudinally cruciately septate, 9–10 $\times$ 7 μ, with slender sterigmata; spores hyaline, even, subglobose, 6 $\times$ 5 μ.

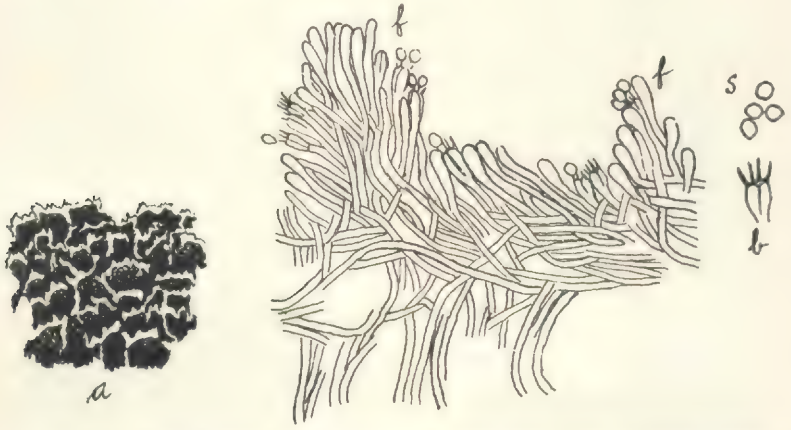

Fig. 1. *P. Farlowii*. *a*, fructification viewed from above, showing pores in black and folds and dissepiments in white, $\times$ 68; *f* and *f*, section of fructification showing pore between folds, $\times$ 375; *b*, basidium, and *s*, spores, $\times$ 375.

Fructifications in small gregarious patches 2–10 mm. in diameter.

On very rotten, decorticated, coniferous wood. New Hampshire. September. Probably very rare.

P. Farlowii should be recognized in the field by its purple color and aspect of *Tulasnella*, and the pruinose surface which is shown by a good lens to have the surface configuration of *Merulius*. The very minute, angular pores, thin and lacerate dissepiments with acute edges, the very small fructifications, and purple color when fresh separate this species from *P. brasiliensis*.

Specimens examined:

New Hampshire: Chocorua, *W. G. Farlow, 6**, type (in Farlow Herb. and in Mo. Bot. Gard. Herb., 55596).

THE MICRO-COLORIMETER IN THE INDICATOR METHOD OF HYDROGEN ION DETERMINATION

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In a recent paper[1] attention was drawn to the use of the colorimeter in determining accurately by the indicator method the hydrogen ion concentration of pigmented biological fluids. In the method there discussed there was employed the well-known principle of compensating for the color of the test solution by introducing the test solution also as a shield solution. It was pointed out that in order to avoid the optical difficulties of the usual tintometers or comparators, while retaining all the advantages of the colorimeter, glass cells fitting one within another are arranged as cup and shield respectively. Properly made these cells are expensive, and the method requires about 15–20 cc. of the liquid for convenient determinations by the colorimeter.

Recently I have had occasion to test the hydrogen ion concentration of some fluids obtainable only in small quantity, and while examining the possibilities of adapting the micro-colorimeter for this work it became clear that the Dubosq type of this instrument lends itself admirably to the colorimetric method in general, and to small quantities of fluid in particular. Moreover, as the method is now modified the necessity for special cells is eliminated. Instead of employing two special cells in connection with each plunger of the colorimeter the principle of the new procedure lies in the use of the plunger tube and of a colorimeter cup as cells on each side of the system. When the quantity of test material available is not limited it is customary in our work to employ 5-cc. quantities, and 5-cc. quantities of the standards, the solutions being prepared in small serological test-tubes to each of which is added 3 drops of indicator. With

[1] Duggar, B. M., and Dodge, C. W. The use of the colorimeter in the indicator method of H ion determination with biological fluids. Ann. Mo. Bot. Gard. 6: 61–70. *f. 1.* 1919.

the micro-colorimeter a quantity as small as 1 cc. of sample may serve both for test solution and for the shield. It is preferable, however, to have not less than 2 cc. for most careful work.

The standardization of the apparatus for this work is extremely simple. It is merely necessary to know the volume of the plunger tube and its length so that in the determinations it will be possible to place a given volume of solution in the plunger cylinder, and knowing the depth which this will occupy, the instrument may be set so that a similar depth will be examined in the colorimeter cup. This is important, since in one case, as described later, the pigmented sample is placed in the plunger tube and in the other case in the colorimeter cup. Therefore equal depths of solutions will be examined in both cells of the systems. In the instrument at our disposal the plunger tube is 33 mm. in length and the volume 1.25 cc. Since the tube is cylindrical the volume is proportional to length, so that if .625 cc. of solution is added the column has a depth of 16.5 mm. The depth need not be so great as this, and 0.5 cc. of liquid is sufficient. If the quantity of the solution employed is reduced beyond this point, it is necessary to increase relatively the amount of indicator added. Where the total quantity of the test solution is 2 cc., 1 cc. being employed for the sample and 1 cc. for the shield, we find it desirable to use 1 drop of indicator for the 1-cc. sample. If this proves too highly colored the indicator may, of course, be diluted one-half.

I find it desirable to arrange the samples, standard, and shields as follows:

> In the left plunger tube place the measured quantity of plain water as shield, and in the colorimeter cup of that side place a quantity of the sample or test solution plus indicator which shall give any depth greater than that of the liquid column in the plunger tube. In the right plunger tube place the measured quantity of the sample as shield, and in the right colorimeter cup the standard solution plus indicator.

With this arrangement it is desirable to make up a few standards covering the range of probability, and then in making the determination it is only necessary to change the solution in the right colorimeter cup until an exact match is obtained. There

is one slight optical defect due to the fact that the surfaces of the liquids in the plunger tubes are not plane surfaces, but this is of no practical consequence in the actual determination, especially where a strong and standard source of light is employed. It is recommended that any of the so-called daylight bulbs be employed in this work. Using the method mentioned no difficulty whatever has been experienced in determining rapidly and effectively the hydrogen ion concentration of such dark liquids as oxidized potato juice, carrot decoction, and decoctions of plants containing considerable chlorophyll.

It is perhaps unnecessary to add that the technique suggested is equally applicable where the large types of colorimeters are employed, such as the Dubosq of standard size, or the Kober (if plunger tubes are detachable), but somewhat larger quantities of solutions will be required.

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STUDIES IN THE PHYSIOLOGY OF THE FUNGI

VIII. Mixed Cultures

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Some consideration has been given in previous literature to the behavior of fungi in mixed cultures, but this has been sufficiently reviewed by Harder,[1] whose work along the line seems to be the most complete. His conclusions, however, are based merely upon observations on the rate of growth and color production in the medium and mycelium. The purpose of his work was to determine whether the inhibition or stimulation of growth, as the case may be, might not be the result of the depletion of the available carbohydrates in the medium or a change in the hydrogen ion concentration.

In the present work the following fungi were used: *Lenzites vialis* Pk., *Merulius pinastri* (Fr.) Burt, *Daedalea quercina* (L.) Fr., *Trametes Peckii* Kalchbr., *Pleurotus sapidus* Kalchbr., *Merulius lacrymans* (Wulf.) Fr., *Lentinus lepideus* Fr., *Daedalea confragosa* (Bolt.) Fr., *Coniophora cerebella* Pers., *Polystictus versicolor* (L.) Fr., *Isaria* sp., *Polyporus lucidus* (Leys.) Fr., *Polystictus hirsutus* Fr., *Aspergillus glaucus*,[2] *A. niger* Van Tieg., *A. fumigatus* Brizi, *A. versicolor* Tiraboschi, and *A. Sydowi* Bainier and Sartory.

All the fungi were grown upon 2 per cent potato agar plates prepared in the manner previously described.[3] After growth

[1] Harder, R. Uber das Verhalten von Basidiomyceten und Ascomyceten in Mischkulturen. Naturwiss. Zeitschr. f. Forst- u. Landw. 9: 129–160. *pl. 3–4. f. 1–2.* 1911.

[2] Thanks are due to Dr. Charles Thom for the determination of the *Fungi Imperfecti* included in this list.

[3] Zeller, S. M., Schmitz, H., and Duggar, B. M. Studies in the physiology of the fungi. VII. Growth of wood-destroying fungi on liquid media. Ann. Mo. Bot. Gard. 6: 137–142. 1919.

TABLE I

Fungus	Organisms stimulating	Organisms inhibiting	Organisms overgrowing	Organisms not influencing
L. vialis	D. quercina*	T. Peckii*, P. sapidus*, Isaria*, A. niger†, A. Sydowi*	T. Peckii, P. versicolor, P. hirsutus	L. vialis, L. lepideus, D. confragosa
M. pinastri		D. quercina†, T. Peckii*, L. lepideus*, D. confragosa†, C. cerebella†, P. versicolor*, P. lucidus*, P. hirsutus*, A. fumigatus†, A. Sydowi*	M. pinastri, T. Peckii, P. sapidus, A. niger	M. pinastri
D. quercina	M. lacrymans*, D. confragosa*, P. lucidus*, P. hirsutus*	M. pinastri†, L. lepideus*, C. cerebella*, Isaria*, A. niger*	T. Peckii, P. sapidus, P. versicolor	L. vialis, D. quercina
T. Peckii	D. quercina*, A. fumigatus*, A. glaucus*	D. confragosa*, Isaria*, A. glaucus*, A. versicolor†, A. Sydowi*	P. sapidus	
P. sapidus		L. vialis*, L. lepideus†, P. versicolor†, P. lucidus*, P. hirsutus†	D. quercina, M. lacrymans	P. sapidus
M. lacrymans		L. lepideus†, P. versicolor†, P. lucidus†, A. fumigatus†	M. pinastri, T. Peckii	
L. lepideus		M. pinastri*, P. sapidus†, P. lucidus†	T. Peckii, P. versicolor	L. vialis
D. confragosa	M. lacrymans†	M. pinastri†, T. Peckii†, P. lucidus†, P. hirsutus†	P. sapidus	L. vialis, A. fumigatus
C. cerebella	L. vialis,* M. lacrymans†	M. pinastri†, D. quercina†, P. versicolor*, A. glaucus*, A. niger*	T. Peckii	

P. versicolor	T. Peckii*	A. versicolor†, A. Sydowi†, M. pinastri*, P. sapidus†, A. niger*, M. lacrymans†, D. confragosa†, C. cerebella†*, P. lucidus†, P. hirsutus†, A. fumigatus*	D. quercina	P. versicolor, A. glaucus
Isaria sp.?		M. pinastri†, A. niger†	P. sapidus, P. versicolor, P. lucidus, P. hirsutus	Isaria
P. lucidus	M. lacrymans*, Isaria*, A. niger*	L. vialis†, M. pinastri*, P. sapidus*, M. lacrymans†, L. lepideus†, D. confragosa†, C. cerebella*, P. versicolor†, A. glaucus†, A. versicolor†	T. Peckii, A. niger	P. lucidus
P. hirsutus		M. pinastri*, P. sapidus†, D. confragosa†, P. versicolor†, A. versicolor*, A. Sydowi*, A. glaucus*	L. vialis, T. Peckii, M. lacrymans, B. cerebella, P. lucidus	P. hirsutus
A. glaucus		T. Peckii*, C. cerebella*, P. hirsutus*, A. glaucus†, A. niger†, A. fumigatus*, A. versicolor†	L. vialis, T. Peckii, P. sapidus, P. lucidus, P. hirsutus	A. fumigatus, A. Sydowi
A. niger		A. versicolor*, A. Sydowi†, D. quercina*, M. lacrymans†, D. confragosa*, C. cerebella*, P. lucidus*, P. hirsutus*, A. glaucus†, A. fumigatus*	T. Peckii, P. sapidus	
A. fumigatus		M. pinastri†, M. lacrymans†, A. glaucus*, A. versicolor†, A. Sydowi†	T. Peckii, Isaria, P. lucidus	D. confragosa, A. fumigatus
A. versicolor		P. hirsutus*, A. fumigatus†, A. glaucus†	P. sapidus, A. Sydowi	P. versicolor, A. glaucus
A. Sydowi		P. hirsutus*, A. niger†, A. fumigatus†	P. sapidus, Isaria, P. lucidus, A. versicolor	

*=after contact; † =before contact.

of the fungi the plates were cut into small squares (about 8 mm. square), which were used as inocula. Agar plates made in a similar manner were each inoculated with three of the fungi in such a way as to have all possible combinations of each fungus. From these plates the reciprocal influence of growth was determined. The results of the plate cultures are shown in table 1.

The outstanding feature of these results is the preponderance of inhibition of growth of one fungus before and after contact with another. In some cases this inhibition took place when the two colonies were still a considerable distance apart; in others only when they came into close proximity with each other. Figures 5 and 2 respectively of pl. 4 illustrate this feature. In those cases where inhibition occurred after contact the condition is shown by a straight line unless one fungus has a much more rapid growth than the other. Figures 8 and 11 illustrate this point. It often happened that one fungus on the plate grew much more rapidly than the other two, cutting off contact between them. Therefore, all possible combinations could not be recorded in the table.

There were not as many instances where one fungous colony grew over another as there were of inhibition of growth. In some cases of the former type one colony was completely covered, and the shape of the submerged colony determined that of the colony of the invading fungus, as, for example, in the case of *Pleurotus sapidus* growing over *Aspergillus glaucus* and *A. Sydowi* (see figs. 3 and 6). In these cases the growth of *Pleurotus sapidus* is greatly accelerated as soon as it reaches the colonies. In other cases the growth of the invading fungus was comparatively slow, as, for example, when *Pleurotus sapidus* invaded a colony of *Aspergillus niger* (see fig. 9). At first both of these fungi were mutually inhibited and then *Pleurotus sapidus* gradually advanced. A peculiarity of this special case is the fact that the spores of *Aspergillus niger* disappeared in the invaded section. It could not be determined whether these spores germinated or were digested.

When two colonies of the same fungus came into contact there was usually no influence of the one colony on the other; that is,

the mycelium of the two thoroughly intermixed, as fig. 12 in the plate shows for *Merulius pinastri*. An exception to this general condition is illustrated when two colonies of *Aspergillus niger* grew together. At first there was an inhibition of growth as shown by a straight line formed by the margins of the two colonies. Later, however, the two colonies generally intermixed. As table I shows, there was often an intermixing of the mycelium of two different species. This may be explained by the theory advanced by Clark[1] that many deleterious substances, which at certain concentrations retard growth, later cause great acceleration of mycelial development in the retarded cultures.

Cases of stimulation of growth when two colonies came into contact were comparatively rare, while cases of stimulation before contact occurred seldom indeed. However, examples of both these types were observed. In many cases it is hard to distinguish between true stimulation and a mere heaping up of the mycelium due to mechanical hindrance. Figures 7 and 10 of the plate show a stimulation of *Trametes Peckii* in contact with *Daedalea quercina*. At first there was a heaping up of the mycelium, and this appeared to be a great stimulation of growth. However, this may equally well be considered a mere increase in the amount of aërial mycelium due to a mechanical hindrance of the surface of the medium. A peculiar case of stimulated growth of *Daedalea confragosa* is shown in fig. 1. It is not certain whether this is caused by the presence of *Merulius lacrymans* or some other factor. However, there does not seem to be any valid reason why a stimulation by diffusion should not be expected as much as inhibition of growth by diffusion, as where *Lentinus lepideus* and *Aspergillus glaucus* are mutually inhibited before contact. In the latter case the colonies never came together. A slight stimulation of growth of *Polyporus lucidus* in the neighborhood of *Isaria* is shown in fig. 4.

It was noticed that the sporulation of certain of the *Fungi Imperfecti* was influenced by the growth of other fungi. For example, there seemed to be an increase in size and number

[1] Clark, J. F. On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. 28: 289–327, 378–404. 1899.

of the heads of conidiospores of *Aspergillus Sydowi* when in contact with *Merulius pinastri*, and the same is true of *Aspergillus niger* in contact with *A. glaucus*.

As previously mentioned, hydrogen ion concentrations of solutions were determined after fungi had grown on them for two weeks, and the results obtained indicate that there is no definite relation between the active acidity produced by these fungi and their ability to inhibit or stimulate the growth of another. For example, in fig. 3, *Pleurotus sapidus* grew over *Aspergillus glaucus* very rapidly, but was entirely inhibited by *A. versicolor* on the solid agar medium. In the solutions *Pleurotus sapidus* produced an active acidity of P_H 5.4, while *Aspergillus glaucus* and *A. versicolor* changed the active acidity to about the degree P_H 6.6 and P_H 6.4, respectively; also, *Trametes Peckii* grew over both *Daedalea quercina* and *Aspergillus fumigatus*, although the change in active acidity produced by *Daedalea quercina* was P_H 3.0 and that produced by *Aspergillus fumigatus* was P_H 6.6. Many such examples could be cited by comparing with table 1 the following active acidities produced by the fungi: *Lenzites vialis*, P_H 5.0; *Merulius pinastri*, P_H 7.0; *Daedalea quercina*, P_H 3.0; *Trametes Peckii*, P_H 4.2; *Pleurotus sapidus*, P_H 5.4; *Merulius lacrymans*, P_H 5.0; *Lentinus lepideus*, P_H 5.4; *Daedalea confragosa*, P_H 5.8; *Coniophora cerebella*, P_H 5.4; *Polystictus versicolor*, P_H 5.4; *Isaria* sp., P_H 6.8; *Polyporus lucidus*, P_H 5.4; *Polystictus hirsutus*, P_H 5.2; *Aspergillus glaucus*, P_H 6.6; *A. niger*, P_H 5.8; *A. fumigatus*, P_H 6.6; *A. versicolor*, P_H 6.4; and *A. Sydowi*, P_H 6.8. The control solution upon which no fungi had grown had an active acidity of P_H 5.4. Of course, there are some instances where similar effects could be correlated with similar changes in hydrogen ion concentration; for instance, *Trametes Peckii* is similarly influenced by both *Aspergillus fumigatus* and *A. glaucus*.

The fungi were also grown on a nutrient solution containing the same ingredients as the agar previously mentioned. Since certain of the *Basidiomycetes* used do not grow well upon liquid media it was found desirable to add to the cultures sufficient quartz sand, free from all soluble substance, so that a slope of sand could be formed above the surface of the solution out into

which the solution diffused. All of the *Basidiomycetes* grew well upon these sand slopes, and the solution was easily drained from the sand at the end of the period of culture. After two weeks' growth of the fungi the hydrogen ion concentration of the solutions was determined according to the methods previously cited.[1]

In cases where there was a marked stimulation or inhibition of growth between two fungi on the plates, these fungi were then grown on similar sand slopes. After they had made considerable growth the solution was filtered off, sterilized, and prepared for inoculation with the reciprocal fungus. Controls of these solutions were kept uninoculated. The amount of growth of the second inoculation was determined by the dry weight of the fungous mat and the amount of sugar remaining in the solutions estimated. The latter was accomplished by reducing equal amounts of the solutions with equal amounts of Fehling's solution and estimating visually the amounts of copper oxide. The distinctions were so evident that quantitative determinations were unnecessary.

The dry weight of mycelium produced in each case and an estimation of the amount of sugar remaining in the solution after growth of the first and second fungus are shown in table II.

In some cases it would seem that the carbohydrate content of the nutrient solution upon which a fungus had previously grown might have been the limiting factor for growth. In others, however, this is not true; for example, when *L. vialis* follows *A. niger* there is very little growth, although the carbohydrate content was high, while in the control solution upon which no fungus had grown *L. vialis* made considerable growth and used a greater part of the sugar in the solution. This would tend to indicate that *A. niger* in its metabolism may have secreted some substance which was toxic to the growth of *L. vialis*. It is of course quite probable that such toxic substances were formed in many more instances but were destroyed in the process of autoclaving between the first and second inoculation.

This in general agrees with the conclusions reached by Fulton[2] that fungi in their growth show a more marked tendency to grow

[1] Zeller, Schmitz, and Duggar, *l. c.*

[2] Fulton, H. R. Chemotropism of fungi. Bot. Gaz. 41: 81–108. 1906.

TABLE II

RELATION OF THE AMOUNT OF GROWTH TO THE QUANTITY OF SUGAR REMAINING IN THE SOLUTION

Fungus	Grown on solution after	Dry weight of mycelium (gms.)	Relative amounts of remaining sugars
L. vialis	A. niger	.080	Much
	A. Sydowi	.062	Trace
	Control	.221	Medium
M. pinastri	T. Peckii	.115	Trace
	A. niger	.040	Trace
	Control	.119	None
T. Peckii	M. lacrymans	.154	Much
	P. sapidus	.335	Much
	Isaria	.140	Medium
	A. niger	.084	Trace
	A. fumigatus	.118	Medium
	A. glaucus	.126	Medium
	Control	.221	Much
P. sapidus	A. niger	.302	None
	A. glaucus	.180	Trace
	A. Sydowi	.309	Trace
	Control	.353	Much
M. lacrymans	T. Peckii	.178	Trace
	D. confragosa	.100	Trace
	Control	.230	Much
L. lepideus	T. Peckii	.094	Medium
	A. niger	.073	Trace
	A. fumigatus	.100	Trace
	Control	.111	Much
D. confragosa	M. lacrymans	.290	Trace
	Control	.231	Much
P. versicolor	P. sapidus	.372	Trace
	A. glaucus	.085	None
	A. Sydowi	.088	Trace
	Control	.266	None
P. lucidus	Isaria	.117	Trace
A. niger	P. sapidus	.270	Medium
	M. lacrymans	.240	Trace
	D. confragosa	.141	Trace
	C. cerebella	.270	Trace
	A. fumigatus	.078	None
	Control	.270	Trace
A. fumigatus	C. cerebella	.156	Much
	Control	.158	Trace

out and *away from* the medium influenced by their own growth metabolism than to grow *towards* a diffusion center, whether this center contains nutritive or deleterious materials. This may also be the condition produced in stale cultures.

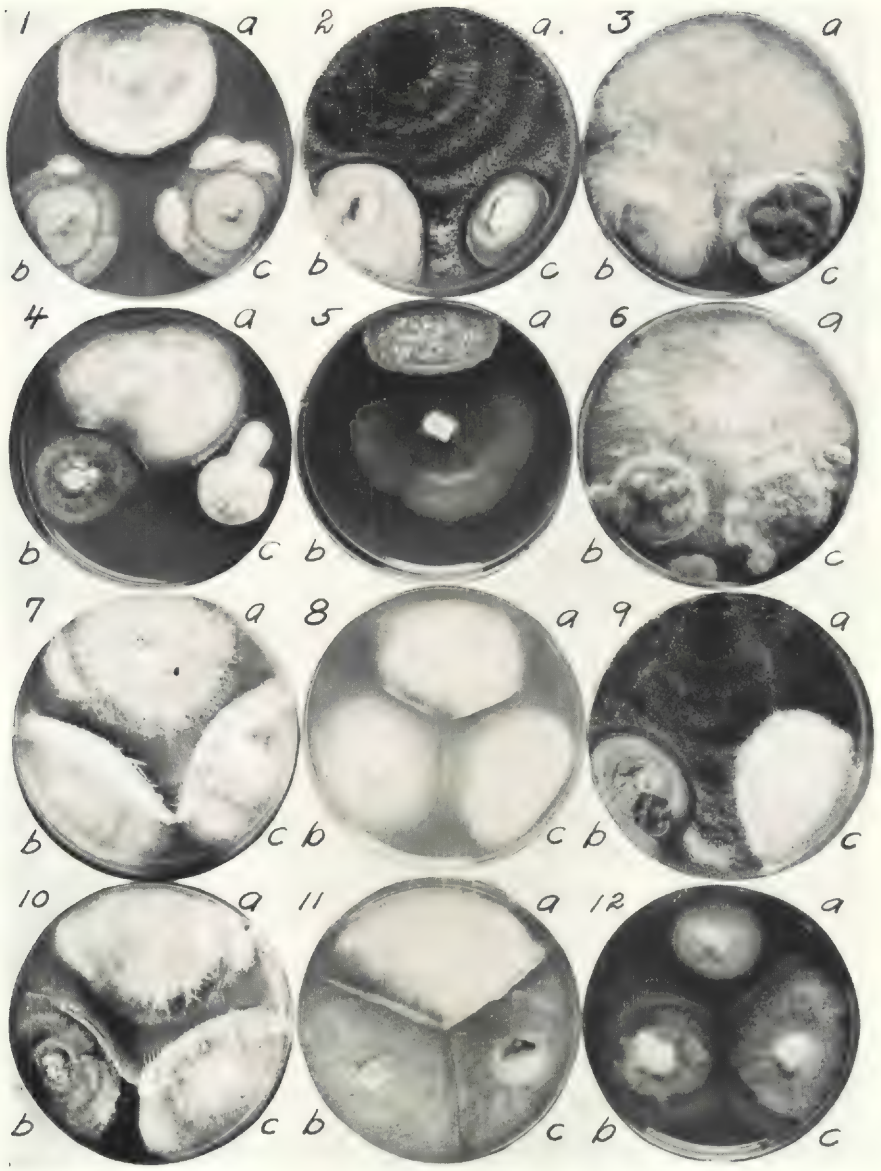
Thanks are due to the Missouri Botanical Garden for the facilities of the library and the laboratories and to Dr. B. M. Duggar for helpful co-operation.

Graduate Laboratory, Missouri Botanical Garden.

Explanation of Plate

PLATE 4

Fig. 1. *a, Merulius lacrymans; b* and *c, Daedalea confragosa.*
Fig. 2. *a, Aspergillus niger; b, A. fumigatus; c, Lentinus lepideus.*
Fig. 3. *a, Pleurotus sapidus; b, Aspergillus glaucus; c, A. versicolor.*
Fig. 4. *a, Polyporus lucidus; b, Lenzites vialis; c, Isaria* sp.
Fig. 5. *a, Aspergillus fumigatus; b, Lentinus lepideus.*
Fig. 6. *a, Pleurotus sapidus; b, Aspergillus Sydowi; c, A. glaucus.*
Fig. 7. *a, Trametes Peckii; b* and *c, Daedalea quercina.*
Fig. 8. *a, Polyporus lucidus; b, Polystictus hirsutus; c, Trametes Peckii.*
Fig. 9. *a, Aspergillus niger; b, A. fumigatus; c, Pleurotus sapidus.*
Fig. 10. *a, Trametes Peckii; b, Lenzites vialis; c, Daedalea quercina.*
Fig. 11. *a, Polyporus lucidus; b, Polystictus hirsutus; c, Merulius pinastri.*
Fig. 12. *a, Daedalea quercina; b* and *c, Merulius pinastri.*



ZELLER AND SCHMITZ – MIXED CULTURES

STUDIES IN THE PHYSIOLOGY OF THE FUNGI

IX. Enzyme Action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr.

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Careful studies in the physiology of the wood-destroying fungi have but recently received the degree of consideration to which their economic importance entitles them. Many economic forms are as yet untouched, and it is our purpose to study some of the fundamental physiological relations existing between fungus and host. The following is the first of a series of investigations concerning especially the enzyme activities of such forms. It is recognized that *Armillaria mellea* has received considerable attention in respect to its physiological relations because of its importance as a root rot of fruit trees, but, as far as the writers are aware, there has been no physiological study of *Daedalea confragosa* or *Polyporus lucidus*, both of which must be recognized as important wood-rotting fungi.

In a recent paper by one of us[1] the literature and methods of enzyme study in the wood-destroying fungi have been sufficiently reviewed, so that in the present paper only specific references to previous literature will be made, and unless otherwise stated, the methods followed will be those previously described.

The fungi from which was obtained the fungous meal used in the present study were grown on sterile, sliced carrot in large Erlenmeyer flasks. While still in an active growing condition the fungous mats were removed and rapidly air-dried by means

[1] Zeller, S. M. Studies in the physiology of the fungi. II. Lenzites saepiaria Fries, with special reference to enzyme activity. Ann. Mo. Bot. Gard. 3: 439–512. *pl. 8–9.* 1916.

of an electric fan. When thoroughly dry the material was finely ground.

ESTERASES

In the study of the esterases of these three fungi methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion were used as substrates. When esterases act upon esters fatty acids are liberated, and thus the concentration of the active acidity can be used as an index of the degree of enzyme action. First, a determination was made of the hydrogen ion concentration of the substrate. A similar determination was also made of the substrate to which a certain amount of autoclaved fungous meal had been added. These two determinations did not always check, due to the introduction of certain substances with the fungous meal and perhaps also to certain buffer effects. The latter determination was taken as the control in each case and compared with a third determination made of the substrate to which a similar amount of fungous meal had been added and incubated twenty-one days.

It was found that there was no apparent esterase activity of any of the fungi on any of the substrates except methyl acetate upon which a slight esterase activity was shown in the case of *Daedalea confragosa* and *Polyporus lucidus*. These results are similar to those found for *Lenzites saepiaria*.[1]

CARBOHYDRASES

The action of carbohydrases was determined upon maltose, lactose, sucrose, raffinose, potato starch, inulin, cellulose from various sources, and hemicellulose. The amount of sugars which reduce Fehling's solution in the enzyme cultures after incubation was taken as the index of enzyme activity. Since this study is merely to indicate the relative activity between the different fungi on the different substrates the results are given as the number of cc. of N/20 potassium permanganate required to oxidize the dissolved copper oxide. The results in the following table are the averages of duplicate enzyme cultures after the Fehling's control had been deducted.

[1] Zeller, S. M., *l. c.*

TABLE I

SHOWING THE ACTION OF CARBOHYDRASES ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

Incubation period	Substrate	P. lucidus			A. mellea		D. confragosa	
		With fungous meal	With fungous meal autoclaved	Without fungous meal	With fungous meal	With fungous meal autoclaved	With fungous meal	With fungous meal autoclaved
		Number of cc. of $\frac{N}{20}$ $KMnO_4$						
14 days	Maltose	36.1	16.3	13.1	36.2	16.3	23.5	14.7
24 days	Lactose	31.7	23.0	18.8	36.9	26.0	24.9	19.3
6 hours	Sucrose	27.5	1.6	1.0	31.3	2.3	11.1	0.3
2 days	Raffinose	32.5	5.2	0.8	12.5	2.6	12.5	0.6
6 hours	Potato starch	19.2	6.1	1.4	25.3	2.6	7.7	1.5
2 days	Inulin	20.3	4.9	0.8	15.4	2.4	7.8	0.9
28 days	Ash cellulose	5.6	0.7	0.3	4.1	0.5	4.3	1.8
28 days	Fir cellulose	5.8	0.3	0.2	3.3	0.4	4.7	0.4
28 days	Oak cellulose	3.8	0.2	0.1	3.6	0.1	3.1	1.3
28 days	Hemicellulose	4.4	0.3	0.1	2.9	0.1	3.2	1.8

In general the carbohydrate activity is greater in *Polyporus lucidus* and *Armillaria mellea* than in *Daedalea confragosa*, with the possible exception of raffinase where the activity in *Daedalea confragosa* approximates that of *Armillaria mellea*. The striking feature of the results is the evident presence of lactase in the three fungi. This is the first record of the presence of lactase in the higher fungi.

In the study of cellulase pure cellulose was prepared from Douglas fir, ash, and red oak, according to the method frequently reported from this laboratory. Suspensions of these in doubly

distilled water were used as substrates. After the enzyme culture had been incubated for 28 days there was a marked increase in the amounts of reducing sugars produced by all the fungi on all of the substrates.

For a hemicellulose substrate cleaned autoclaved endosperms from date seeds were used. These were shaved into very thin slices and placed in distilled water with the fungous meal. Hydrolysis quite comparable to that produced in the cellulose experiments resulted in each case as is indicated in table 1.

EMULSIN

The presence or absence of emulsin was determined by the effect of the fungous meal upon amygdalin, which upon hydrolysis produces glucose, benzaldehyde, and hydrocyanic acid. After incubation of seven days the amount of glucose present in the cultures was determined as in the cases where carbohydrates were used as substrates, and the results are tabulated in the following table:

TABLE II

SHOWING THE ACTION OF EMULSIN ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

Enzyme culture	P. lucidus	A. mellea	D. confragosa
	Number of cc. of $\frac{N}{20}$ $KMnO_4$		
1% amygdalin+ fungous meal	26.0	12.5	18.1
1% amygdalin+ fungous meal (autoclaved)	4.8	0.6	0.1
1% amygdalin	0.6	0.6	0.6

In all cases where the above sugar tests showed evidence of the breaking down of amygdalin the odor of benzaldehyde was easily recognized. There was evidence of emulsin in all three of the fungi used.

TANNASE

In order to determine the tannase activity the gallic acid, which is a product of hydrolysis of tannic acid, was titrated with

standard iodine solution. The results show the presence of tannase in *Polyporus lucidus* and *Daedalea confragosa* but none was detected in *Armillaria mellea*. This fact seems peculiar, since the rhizomorphs of *Armillaria mellea* are usually found next to the inner bark of the woody tissues where the tannin is usually present in the greatest amount for the specific host. The fact that the fungi from which the fungous meal was made were grown on carrot may have had some influence on the production of tannase in this particular instance.

Amidase and Urease

The presence of the enzymes which split amino acids and urea into ammonia and hydroxy acids was demonstrated by using such substrates as asparagin, acetamid, and urea. The usual Folin method of determining the presence of ammonia is such a time-consuming procedure that a new method was devised involving the indicator method of determining the hydrogen ion concentration of solutions. In brief, the method employed is as follows:

The substrate and fungous meal in the desired proportions were placed in wash bottles, the inlets and outlets of which were sealed with rubber tubes and clamps in order to retain any ammonia which might have been given off during the period of incubation. After a period of incubation of seven days the ammonia was drawn directly through another small wash bottle by means of a Richards pump. The small wash bottle contained 10 cc. of doubly distilled water to which was added 6 drops of brom thymol blue made up in the proportions suggested by Clark and Lubs.[1] This doubly distilled water had a hydrogen ion concentration of P_H 5.6, at which concentration the indicator was yellowish brown. Due to the hydrogen ion concentration decreasing as the ammonia is drawn through, the color changes from green to blue. The length of time taken to change from P_H 5.6 to P_H 7.0 would, of course, depend upon the amount of ammonia present, and this was thus taken as a criterion of the

[1] Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact. 2: 1–34, 109–136, 191, 236. 1917.

relative rate of ammonia production in the various enzyme cultures. In no case was the gas drawn through the wash bottle for a period longer than three minutes. At the end of this period the actual hydrogen ion concentration of the distilled water was determined. Sometimes the change was so rapid that it was not necessary to run the experiment for three minutes. In such instances other indicators having a wider alkaline range were substituted for brom thymol blue. In the urea control there was a change from P_H 5.6 to P_H 6.0, and thus, for urea, changes not going beyond P_H 6.0 were considered as negative. The results are tabulated in table III.

Urease was demonstrated for the three fungi. It was most pronounced in *Daedalea confragosa* and least in *Polyporus lucidus*. Only *Armillaria mellea* showed slight amidase action when acetamid was used as a substrate. There was no amidase action when asparagin was used as a substrate.

Due to the fact that traces of alkalis or acids cause considerable shifting of the hydrogen ion concentration in such an unbuffered solution as doubly distilled water it is believed that this method can be used to determine the presence of minute traces of ammonia which would be undetectable by the methods usually employed, and the determination is much more rapid. In the present paper only relative determinations were necessary but there is no valid reason why quantitative determinations could not be made by this method.

PROTEASES

Tryptic and ereptic fermentation was studied by the use of albumin, peptone, casein, legumin, and fibrin in enzyme cultures having a neutral, acid, and alkaline reaction. When fibrin was used as a substrate positive results were obtained to show the presence of both trypsin and erepsin in all three of the fungi. These results were most pronounced in the cultures having an acid reaction and least in those with an alkaline reaction. In the case of the plant protein, legumin, there was very slight indication of the presence of tryptic and ereptic fermentation only in *Polyporus lucidus* and only when the substrate was acid in reaction. In *Polyporus lucidus* there was indication of the

presence of trypsin when albumin of acid reaction was used. This was not true of *Armillaria mellea* or *Daedalea confragosa*. In no case was there a splitting of peptone or casein.

TABLE III

UREASE AND AMIDASE ACTIVITY IN ARMILLARIA MELLEA, DAEDALEA CONFRAGOSA, AND POLYPORUS LUCIDUS

	Enzyme culture	No.	Change in H ion concentration. P_H values		
			Urea	Acetamid	Asparagin
P. lucidus	Substrate + fungous meal	1	5.6–6.8 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–6.6 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–5.8 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
A. mellea	Substrate + fungous meal	1	5.6–7.2 30 sec.	5.6–6.0 3 min.	5.6–5.6 3 min.
		2	5.6–7.2 25 sec.	5.6–6.0 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
D. confragosa	Substrate + fungous meal	1	5.6–7.2 1 sec.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–8.8 3 sec.	5.6–5.6 3 min.	5.6–5.6 3 min.
	Substrate + fungous meal (autoclaved)	3	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		4	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
Control	Substrate alone	1	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.
		2	5.6–6.0 3 min.	5.6–5.6 3 min.	5.6–5.6 3 min.

Summary

In *Polyporus lucidus* the presence of the following enzymes is demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

In *Armillaria mellea* the presence of the following enzymes is demonstrated: maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, urease, amidase, and trypsin and erepsin when fibrin is used as a substrate.

In *Daedalea confragosa* the following enzymes are present: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

A new method for the determination of ammonia liberated by amidase is described. This method involves the application of the indicator method for hydrogen ion concentration determination.

Thanks are due to the Missouri Botanical Garden for the privileges of the library and laboratories and to Dr. B. M. Duggar for advice and helpful criticisms.

Graduate Laboratory, Missouri Botanical Garden.

STUDIES IN THE PHYSIOLOGY OF THE FUNGI

X. Germination of the Spores of Certain Fungi in Relation to Hydrogen Ion Concentration[1]

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Introduction

The hydrogen ion concentration of culture media or solutions has come to be regarded in recent years as one of the most important factors influencing physiological phenomena. A voluminous literature is found dealing with the toxic properties of H and OH ions in a general way, but the earlier investigators, like many of the later ones, were handicapped by lack of methods, or experience with methods, for the direct determination of hydrogen ion concentration. With such limitation in technique, conductivity data have frequently been employed in the interpretations made. This method is, however, inapplicable when other solutes are introduced, and the presence of strong buffers, whether inorganic or organic, would render most difficult any computation of active acidity or alkalinity.

Some of the questions which are unanswered are: What is the effect of hydrogen ion concentration upon the rate of germination of the spores of certain fungi, or, what is the range within which the most favorable germination occurs? Such questions suggested the desirability of conducting the investigation reported in this paper, and the scarcity of definite literature dealing with this particular phase has been one of the greatest incentives to the pursuance of the problem.

Review of Literature

Clark ('99) seems to have been one of the pioneer workers on the toxicity of acids, alkalis, oxidizing agents, and salts of the heavier metals, towards the growth of certain fungi. Using the

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of master of arts in the Henry Shaw School of Botany of Washington University.

hanging-drop method, he determined, in a nutrient medium, approximately the relative and absolute toxic properties of many deleterious agents as shown by their influence on spore germination, mycelial development, and fructification. The medium used throughout this study was an infusion of sugar beet, as experiments have shown this to be the most suitable and satisfactory medium for all the forms. The toxicity of the various acids and alkalis towards moulds is shown in detail, and interest centers upon (1) the average inhibiting concentration for germination and development, and (2) the average killing concentration, the organisms employed being *Aspergillus flavus*, *Sterigmatocystis nigra*, *Oedocephalum albidum*, *Penicillium glaucum*, and *Botrytis vulgaris*.

It was a general rule with all acids and alkalis that when the concentrations were not sufficient to cause distinct injury, stimulation of growth followed the slight retardation, such cultures taking on new vigor and surpassing the controls. Spores of *Botrytis* were most easily killed, while those of *Penicillium* offered the greatest resistance. Comparing the results with conductivity data, Clark concludes that the OH ion is more toxic towards fungi than the H ion. It is to be remembered, however, that the hydrogen ion concentration of none of his solutions was known. Nevertheless, this is of special interest when taken in conjunction with data which I shall present later.

Using distilled water as a medium, Stevens ('98) studied the effect of salts, bases, and acids upon the germination of the following fungous spores: *Botrytis vulgaris*, *Macrosporium* sp., *Penicillium crustaceum*, *Gloeosporium musarum*, and *Uromyces caryophyllinus*. As might be expected, germination of most of these species is not perfect in distilled water or else there is considerable variability in the results. Abnormal and distorted mycelium appear more frequently in the acid solution than in ordinary nutrient media. With *Macrosporium* and *Penicillium* neither HCl nor H_2SO_4 prevented growth, and the behavior of *Uromyces* towards these acids was quite variable. Further work with alkalis tends to indicate that KOH, NaOH, and NH_4OH have a low toxic value. *Penicillium* generally offered the greatest resistance to the different agents. From the data obtained,

the author concludes that various fungi exhibit varying degrees of resistance to poisons, and that the limits of resistance may vary in the species. Even though little data were at hand, Stevens concluded that the spores of fungi, when compared to the roots of seedlings, were less susceptible to toxic action.

Duggar ('01) made an extensive study of spore germination, including certain chemical as well as physical stimuli. Using distilled water as the medium, he found that organic acids stimulated germination but the percentage of germination was not great. The stimulus of N/100 or less of acetic acid to *Aspergillus flavus* and *Sterigmatocystis nigra* was very noticeable. Oxalic acid was more pronouncedly stimulating with *Sterigmatocystis*, N/100 producing maximum germination, whereas this concentration totally inhibited germination of *Aspergillus*. In this work, the considerable extent of individual variation was emphasized.

Ferguson ('02), using an artificial digestive fluid containing solutions of pepsin in distilled water combined with different amounts of HCl also in distilled water, studied the germination of spores of *Agaricus campestris*, *Coprinus comatus*, and *Calvatia cyathiforme*, but germination was so erratic that she was unable to draw any definite conclusions.

Brooks ('06) studied the effect of temperature on the toxic properties of $CuSO_4$, HNO_3, and H_2SO_4, as shown by the effect of these substances on the germination and growth of certain fungi. In all the experiments, beet decoction was used as the nutrient medium, the stock infusion containing 600 gms. of beets per 1000 cc. of water. At the time of using, the decoction was diluted by the addition of the toxic substance and water to one-half of its former nutritive value. Usually, above the provisional optimum, the deleterious action of the toxic agents increased very rapidly with rise in temperature. Spores inhibited by cold were not greatly injured when exposed to harmful agents. In all instances, however, the injurious effects were least at the optimum temperature for the fungus; however, the effects of the three chemicals were very different.

Ayers ('16), making cultures of streptococci in a broth containing 1 per cent cerevisine, 1 per cent peptone, 1 per cent test

substance (glucose, lactose, etc.), and distilled water, obtained data indicating that streptococci reach more or less definite hydrogen ion concentration and that there are two limiting zones, P_H 4.6–4.8 and P_H 5.5–6.0. A very large percentage of the streptococci from cases of human infection reached only the lower limit of hydrogen ion concentration, a fact that is very striking.

Morgan and Gruzit ('16) found that soil solutions adjusted to various reactions by N/100 mineral acid and N/100 alkali, when mixed with sterile quartz, showed variations in the type and number of bacteria. A solution with N/1000 alkali gave the best growth while N/1200 acid exerted marked toxicity. In alkaline solutions the number of bacteria increased up to the point of faint alkalinity and then decreased after passing this point; whereas in acid solutions the number of bacteria increased with decrease in acidity.

Zeller ('16) found that the reaction of the medium was a most important factor influencing the growth and metabolism of *Lenzites saepiaria*. A medium of Thaxter's glucose-potato-hard agar possessing the faintest alkalinity failed to produce the slightest growth, but, on being readjusted to slight acidity, it gave good growth. Spaulding ('11) found that the same organism was unusually sensitive to alkaline media, and obtained luxuriant growth with one-fourth of 1 per cent H_2SO_4. Other investigators have published similar results.

Salter ('16) found that the reproduction of legume bacteria in Ashby's mannite solution and in a soil solution was greatly influenced by the reaction of the medium. A neutral or slightly acid reaction in mannite solution, the means of determination of which are not stated, proved to be the most favorable for the production of the red clover organism. Inhibition of growth was evident in slightly alkaline solutions, and no growth was found in the presence of 1 per cent normal alkali. *Bacillus radicicola* from alfalfa, on the other hand, exhibited great sensitiveness towards acidity, retardation of growth being noticed with .5 per cent normal H_2SO_4. The organism grew best in faintly alkaline or neutral mannite solution.

Clark and Lubs ('17) grew *Aspergillus niger* on a medium con-

sisting of 1 gm. KH_2PO_4, 3 gms. $NaNO_3$, .5 gm. $MgSO_4$, 100 gms. sucrose in 1 liter water, and on the seventh day found the hydrogen ion concentration to be 2×10^{-2}. They comment upon Waterman's estimate that the critical limit for *Penicillium glaucum* is about 1×10^{-5} N. H. and for *Aspergillus niger* about 4.5×10^{-5} N. H. The reviewers think that the only explanation for such discordant results must lie in a confusion in the method of expressing hydrogen ion concentration.

Fred and Loomis ('17) found that a mannitol solution with a neutral reaction gave the highest count of *B. radicicola* from alfalfa. The addition of small amounts of alkali did not appreciably alter the number of bacteria; however, acid in equivalent amounts either retarded or inhibited growth. From the curve of hydrogen ion concentration, they are inclined to think that the apparent resistance of the legume bacteria to alkali is due to the slight concentration of hydroxyl ions in the mannitol solution. This work confirms that of Salter.

Gruzit ('17) studied the effect of acids and alkalis on soil bacteria in soil solution, and found that soil bacteria were extremely sensitive to an acid reaction. H_2SO_4 at a concentration of N/1200 destroyed about 99.6 per cent of the bacteria; N/1400 killed about 93.0 per cent of the organisms; and N/2840 prevented the growth of about 43.0 per cent. On the other hand, N/1000 alkali gave the maximum number of bacteria.

Taylor ('17) determined the concentrations of a few organic and inorganic acids necessary to check the growth of various organisms. He obtained data which led him to conclude that there is a great variation or specificity in their activity toward different organisms.

Wolf and Harris ('17) observed that the acidity of the medium may either delay or entirely stop the growth of *B. perfringens* and *B. sporogenes*, the critical concentration of the former being P_H 4.82 and the latter, P_H 4.94. All the acids tested gave very similar effects and showed practically no specific qualities.

Wright ('17) studied the importance of uniform culture media, and obtained data which clearly emphasize the many discrepancies that exist when the culture medium is adjusted by means of phenolphthalein titration. He found that the hydrogen ion

concentration of the culture medium and the resistance of organisms to the action of disinfectants afford a definite relation, the greatest resistance being obtained with a culture medium having a hydrogen ion concentration P_H 6.0–7.0.

Fred and Davenport ('18) found that the growth of the nitrogen-assimilating bacteria in culture solutions of different reactions was related to the hydrogen ion concentration of the medium. Of the legume bacteria, the organisms of alfalfa were the most sensitive to hydrogen ion concentration, the limit of growth on the acid side being between P_H 5.4 and 5.6; while, on the contrary, the organisms of lupine were the most resistant, the limit of growth on the acid side being P_H 4.6. Sodium hydroxide did not cause any noticeable toxicity towards the legume bacteria until added in greater quantities than N/125 and appeared to have only one-tenth the toxic properties of H_2SO_4 towards these organisms. The authors cite Beijerinck as having secured optimum growth of *Rhizobium leguminosarum* in N/166.6 acid, but explain the disagreement of results on the ground of employing different culture media. *Azotobacter* proved to be very sensitive to slight changes of reaction and was able to grow only within the narrow limits, P_H 6.5–8.6.

Meacham ('18) determined the hydrogen ion concentration of synthetic and malt-extract media necessary to inhibit the growth of *Lenzites saepiaria*, *Fomes rosens*, *Coniophora cerebella*, and *Merulius lacrymans*. Growth is not inhibited until a very high hydrogen ion concentration is reached, and, while the different fungi show considerable fluctuations, the organisms respond in much the same way. In general, growth proceeds in a straight line until about P_H 2.6; decreases almost abruptly at P_H 2.6, the range P_H 2.6–1.9 being termed the "critical range"; from P_H 1.9, the decline is more gradual and the limiting P_H value appears to be about 1.7. Prior to the sudden decrease at P_H 2.6, there frequently occurs a maximum of growth, usually about P_H 3.0.

Krönig and Paul ('97) found a solution of HNO_3 to be distinctly more toxic to anthrax spores than the same concentration of HCl. Their results with acetic acid were similar to those obtained by Clark ('99), but the results with alkalis were not consistent with Clark's.

Methods

The methods employed in this investigation, as described by Clark ('99) and Duggar ('01), are substantially those used by others in this laboratory.

Organisms.—The fungi used were *Aspergillus niger*, *Penicillium cyclopium*, *Fusarium* sp., *Botrytis cinerea*, and *Lenzites saepiaria*. An attempt was made to use *Colletotrichum lindemuthianum*, but, owing to the failure to obtain germination in the control culture solutions, this organism was discarded. In the test-tube cultures from which the spores were obtained, the fungi were grown on potato agar made according to Duggar, Severy, and Schmitz ('17); i. e., 230 gms. of potato were cut into small pieces, autoclaved in 1 litre of water for 1 hr. at 15 lbs. pressure, filtered while hot, 15 gms. of agar added, the mixture then autoclaved for 15 minutes at 15 lbs., correction made for loss of water, and finally tubed, sterilized, and slanted. The cultures were allowed to grow at room temperature, and the spores were always taken from cultures that were from 10 to 15 days old.

Culture solutions.—The composition of the culture solutions was based primarily on Clark and Lubs's ('17) titration curve of ortho-phosphoric acid. Stock solutions of M/5 mannite in M/10 H_3PO_4 and M/5 mannite in N/5 NaOH were made. Into sterile Pyrex flasks, 100 cc. of the M/5 mannite-M/10 H_3PO_4 solution were placed, and increasing proportions of M/5 mannite-N/5 NaOH were added. The flasks were plugged with cotton and sterilized at 15 lbs. pressure for 15 minutes, after which the hydrogen ion concentrations were determined by the colorimetric method as outlined by Clark and Lubs ('17). The procedure was as follows:

To a test-tube containing a 10-cc. portion of the culture fluid the proper indicator was added, and the color developed compared with the colors obtained upon the addition of the same indicator to tubes containing equal quantities of standard buffer solutions. All solutions were made from the best chemicals, purified according to Clark and Lubs ('17), and made up with doubly distilled water. A series of solutions was thus obtained ranging in hydrogen ion concentration from P_H 2.8 to 10.0+. In nearly every case the determined value was identical with

the calculated value, the greatest divergence being .2. In the alkaline range the concentration of the OH ions in the last solution was beyond the range of the indicator, so it has been designated as 10.0+. The ten solutions, termed a series, are as follows: P_H 2.8, 3.1, 4.4, 5.0, 6.2, 7.0, 7.4, 8.8, 9.6, and 10.0+.

Small portions of each of the solutions were transferred to sterile test-tubes permanently labeled and fitted with rubber stoppers, through each of which passed a glass rod drawn to a blunt point. All transfers of a solution were made with its particular rod, thus avoiding all chances of mixing solutions. Fresh solutions were placed in the tubes from time to time, and verifications of hydrogen ion concentration frequently made.

Method of culture.—The method of culture was based primarily on the hanging-drop or Van Tieghem cell. The glass cylinders employed were perfectly ground at each end and measured 18.0 mm. in diameter and 9.0 mm. in height, possessing therefore a volume of 2.3 cc. The cylinders were cemented to the slide by means of wax; the tops of the cylinders were then coated with a thin ring of vaseline, and the cells completed by sealing cover glasses to the tops of the cylinders. A small nick was made in each ring of vaseline, prior to sealing, so that equilibrium of air pressure might exist when the cultures were placed in the incubator. About 15 minutes later, the cultures were examined and the cover glasses slightly pressed to the cylinders in order to insure a perfect sealing. Two cells were placed on each slide and labeled by gumming numbered and lettered labels to the center of the slip.

Four or five drops of the same solution as that to be used in the culture were placed in the bottom of the cell, the object being to establish a complete equilibrium of vapor pressures in the cells and to prevent changes in the concentration of the solution tested, as shown by Clark ('99). A few drops of the same solution were also placed on a sterile slide, and spores transferred from a pure culture to the slide by means of a sterile platinum needle. A solution of spores was thus made and thoroughly stirred in order to prevent the spores from adhering in bunches. A drop of the spore solution was transferred from the slide to the cover glass by means of a clean,

sterile, glass rod drawn to a small point. The cover glass bearing the culture was then inverted on the cell and gently pressed until completely closed with the exception of the minute opening previously described. All cultures were made up at room temperature, and, when a set of cultures was completed, all were placed in an incubator and kept at a constant temperature. Cultures of each organism were incubated at 22° C., 27° C., and 31° C., respectively, with the exception of *Lenzites saepiaria*.

Care of cells, etc.—The glass rings and slide composing the cells were never used a second time without being taken apart and thoroughly cleaned by boiling in alkali, soaking in cleansing mixture, and repeatedly rinsed in distilled water. They were then sterilized in an oven at 150° C. for 1 hour and protected from dust until needed. The cover glasses were treated similarly except that they were boiled longer in order to remove all traces of vaseline, and finally dipped in alcohol and wiped dry. All slides, rods, etc., were placed in water after having been used, and given the same general treatment before setting up another series.

Examination of cultures and data.—Cultures were made up, as a rule, in the afternoon and examined at different intervals, depending on the length of time required for the spores of a particular organism to germinate, as determined by a preliminary experiment. Spore counts were made from five fields of the hanging drop, and the average percentage of germination recorded. Where possible three different readings were made with each set of cultures, but often the mycelial growth was so luxuriant that only two were possible. All of the experiments were run in duplicate, and the germination data reported in this paper represent the percentage averages of the two cultures. Although slight fluctuations occurred throughout the work, the result from any cell agreed very closely with that of the duplicate.

Curves.—The curves are developed from the percentage averages, as indicated above, each curve representing the final reading of the germination quantities of a certain organism at a particular temperature. The percentages of spore germination are plotted as ordinates and the hydrogen ion concentration of

the solutions as abscissae. Curves corresponding to each of the temperatures of incubation are found in each figure, the solid line representing 22° C., the dotted line, 27° C., and the broken line, 31° C.

EXPERIMENTAL DATA

In examining the experimental results, it must be borne in mind that perfect germination is not to be expected with these fungi in a solution containing mannite as the sole nutrient. In fact, dextrose would perhaps have yielded higher germination percentages, but it is not certain that it would remain stable with the treatment given. Moreover, in this preliminary work it was not desired to employ a full nutrient solution on account of greater difficulties of P_H adjustment.

The influence of hydrogen ion concentration upon the germination of the spores of *Aspergillus niger* may be seen by referring to table I.

TABLE I

ASPERGILLUS NIGER. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

Temp.	Hrs.	Hydrogen ion concentration, P_H								
		2.8	3.1	4.4	5.0	6.2	7.0	7.4	8.8	9.6
22° C.	16	33.1	64.2	33.4	19.8	13.7	9.0	2.0	0.0	0.0
	26	63.5	83.1	55.0	42.1	18.1	13.4	14.0	8.2	0.0
	38	66.8	87.5	55.6	42.9	20.2	15.5	15.8	8.6	0.0
27° C.	16	45.3	69.4	25.9	20.0	5.5	15.1	0.0	0.0	0.0
	26	53.0	71.8	31.0	23.7	7.1	20.1	0.0	0.0	0.0
31° C.	16	22.6	32.6	46.6	44.0	24.4	18.6	0.0	0.0	0.0
	26	28.1	50.4	47.7	45.4	32.0	22.6	3.0	0.0	0.0

Incubated at 22° C., the data show that maximum germination is obtained in the culture having a hydrogen ion concentration of P_H 3.1. With further increase in hydrogen ion concentration, there is a marked inhibition of germination, the percentage decreasing from 64.2 at P_H 3.1 to 33.1 at P_H 2.8; while, with decrease in hydrogen ion concentration from P_H 3.1, there is a

general decrease in percentage of germination. Only a comparatively small amount of germination is obtained in the culture testing P_H 7.4 and no germination whatever is evident at P_H 8.8. The limiting concentration of hydrogen ions lies between P_H 7.4 and 8.8 on the alkaline side, and below P_H 2.8 on the acid side.

Examination of the same series after an incubation of 26 and 38 hours, respectively, shows that germination has increased in all the cultures and that there have been several slight changes in the curve of germination. A slight maximum is noticed on the alkaline side at P_H 7.4, and a relatively low percentage of germination is obtained at P_H 8.8, which at 16 hours was the limiting concentration.

A series incubated at 27° C. for 16 hours gives a general curve of germination very similar to that incubated at 22° C. with the exception of several slight shifts. A maximum of germination is obtained at P_H 3.1, as before, but the limiting concentration on the alkaline side shifts toward neutrality; thus even at P_H 7.4 there is total inhibition of germination, as compared with P_H 9.6 in the first series. With decrease in hydrogen ion concentration from P_H 3.1 there is a general decrease in percentage of germination to culture P_H 6.2 where a minimum is reached, followed by a relatively small rise at P_H 7.0. Upon incubating the series for 10 additional hours, the same relations of germination are obtained. On account of the luxuriant mycelial growth, only two readings were possible with this series.

Incubation at 31° C. gives slightly different results from those incubated at 27° C. At the time of the first reading the maximum count occurs at P_H 4.4, and there is no evidence of stimulation of germination in the neutral or slightly alkaline cultures. On the other hand, germination decreases with decrease in H ion concentration from P_H 3.1 to 7.0, the limiting concentration proving to be P_H 7.4. In the final reading maximum germination on the acid side shifts to P_H 3.1, thus making the maximum germination at each temperature occur at P_H 3.1; and the remaining figures confirm the results of the previous examination.

These relations are shown graphically in the curves of fig. 1.

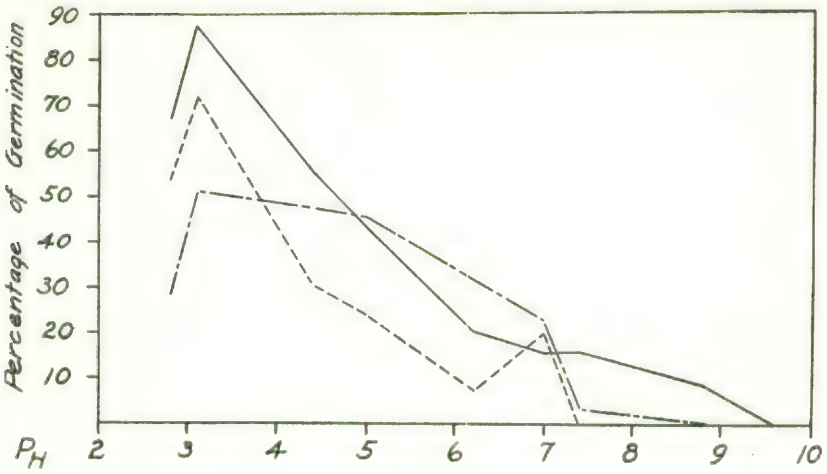

Fig. 1. *Aspergillus niger.* Graphic representation of the relation of germination to H ion concentration.

The data obtained with spores of *Penicillium cyclopium* as given in table II, are somewhat similar to those with *Aspergillus niger*.

TABLE II

PENICILLIUM CYCLOPIUM. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

Temp.	Hrs.	Hydrogen ion concentration, P_H									
		2.8	3.1	4.4	5.0	6.2	7.0	7.4	8.8	9.6	10.0+
22° C.	18	4.1	13.5	16.8	14.0	7.1	6.6	10.5	4.4	2.1	0.0
	27	10.3	21.1	30.7	16.6	10.9	8.1	28.7	10.5	2.7	0.0
	37	11.4	22.7	32.3	23.6	11.9	8.6	31.9	13.0	3.1	1.7
27° C.	18	32.2	35.5	22.9	20.7	10.5	12.3	7.0	0.0	0.0	0.0
	27	58.6	65.6	52.2	32.5	18.1	28.7	12.1	0.0	0.0	0.0
	37	59.8	66.6	55.4	35.1	22.0	33.2	18.7	0.0	0.0	0.0
31° C.	18	15.6	44.2	29.3	13.4	9.7	5.6	9.6	0.0	0.0	0.0
	27	30.0	57.8	39.3	25.2	19.5	6.8	12.1	5.0	0.0	0.0

When incubated at 22° C. for 18 hours, maximum germination is obtained in the culture with a hydrogen ion concentration of P_H 4.4, and percentage germination decreases with decrease in hydrogen ion concentration to the culture testing P_H 7.0 where

minimum germination is obtained. At P_H 7.4 there is a slight increase followed by a gradual decline to the culture possessing an exponent 9.6, the limiting concentration appearing to be beyond 10.0+. With increase of hydrogen ion concentration above P_H 4.4, there is a decrease in percentage of germination, but the limiting concentration evidently lies above the concentration P_H 2.8. Readings at incubation periods of 27 and 37 hours, respectively, give very similar results to those of the first reading, the only difference being that relatively slight germination is obtained in the culture with the value P_H 10.0+, which was formerly the limiting concentration. In no other case did *Penicillium* germinate at this relatively extreme alkalinity, and, inasmuch as these spores frequently collected in bunches, it is thought that the apparently erratic germination might be due to this fact.

At 27° C. maximum germination is obtained in the culture where the exponent is P_H 3.1, and the curve proceeds in the same general direction as before with the exception that minimum germination is obtained at P_H 6.2, as compared with P_H 7.0 in the former case. A slight rise is evident at P_H7.0, only to be followed by a decline at P_H 7.4. On the acid side, the limiting concentration occurs below P_H 2.8, whereas on the alkaline side it occurs about P_H 8.8. Both examinations of prolonged incubation substantiate the data obtained from the first reading, the only difference being a gradual increase of germination with increase of incubation interval.

The same curve of germination is obtained at 31° C. The characteristic maximum occurs in the same culture as before, exhibiting the value P_H 3.1; germination decreases with decrease in hydrogen ion concentration to the culture testing P_H 7.0 where the minimum is obtained. A slight stimulation in germination is noted at P_H 7.4, but no germination whatever is noticed with further decrease in hydrogen ion concentration. With an additional incubation of nine hours at the same temperature, germination relations remain practically the same. The range of germination is extended to P_H 8.8, as compared with P_H 7.4 at the first examination, and germination is extremely low at P_H7.0. Mycelial growth was so luxuriant in this series that it was

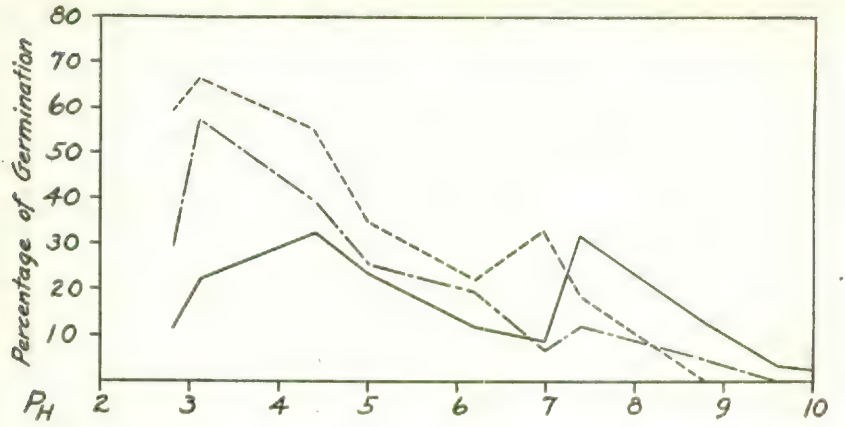

Fig. 2. *Penicillium cyclopium*. Graphic representation of the relation of germination to H ion concentration.

impossible to make a third reading. These relations are shown graphically in fig. 2.

An acid reaction decidedly favors spore germination of *Botrytis cinerea*, as seen by referring to table III.

TABLE III

BOTRYTIS CINEREA. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

Temp.	Hrs.	Hydrogen ion concentration, P_H									
		2.8	3.1	4.4	5.0	6.2	7.0	7.4	8.8	9.6	10.0+
22° C.	6	70.0	86.6	63.4	52.6	48.2	22.3	0.0	0.0	0.0	0.0
	21	77.1	90.2	72.9	62.6	57.8	46.0	0.0	0.0	0.0	0.0
27° C.	6	42.0	32.2	50.0	22.3	15.3	0.0	0.0	0.0	0.0	0.0
	21	92.4	66.4	61.2	34.9	30.1	0.0	0.0	0.0	0.0	0.0

Incubated at 22° C. no germination is obtained at P_H 7.4; little germination is obtained at P_H 7.0; and, with increasing hydrogen ion concentration, the germination quantities increase until a crest is reached in the culture with P_H 3.1. With further increase of hydrogen ion concentration, there is a diminution in percentage of germination. The data obtained after an incubation period of 21 hours are very consistent with those of the

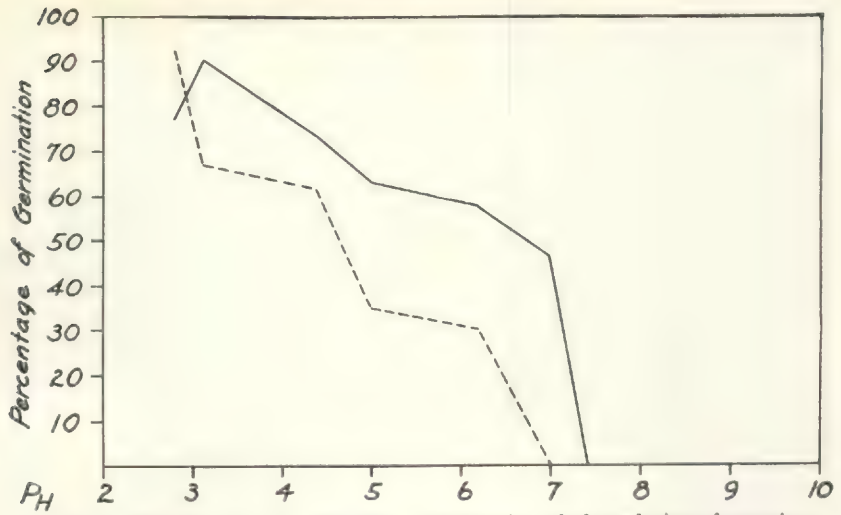

Fig. 3. *Botrytis cinerea.* Graphic representation of the relation of germination to H ion concentration.

6-hour period. Germination at 27° C. proved very similar to that at 22° C., except for the fact that the entire curve appears to have shifted one remove towards the acid side.

A series was made up and incubated at 31° C., but frequently the temperature went as high as 31.5° C. In no culture was there any sign of germination. This incidental datum is in accord with the results obtained by Duggar ('01). He found that a temperature of 32° C. was distinctly injurious to spores of *Botrytis.* Figure 3 exhibits the curves of germination at each of the successful temperatures.

TABLE IV

FUSARIUM SP. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

Temp.	Hrs.	Hydrogen ion concentration, P_H									
		2.8	3.1	4.4	5.0	6.2	7.0	7.4	8.8	9.6	10.0+
22° C.	6	0.0	0.0	7.2	9.5	11.6	11.7	65.3	21.3	8.5	24.6
	20	57.8	38.0	21.9	20.7	16.0	20.1	67.7	36.6	27.7	26.9
27° C.	6	0.0	9.7	31.0	45.4	18.0	23.3	50.0	23.7	5.1	0.0
	20	7.9	65.8	59.6	56.9	33.3	39.6	66.3	24.7	15.6	7.9

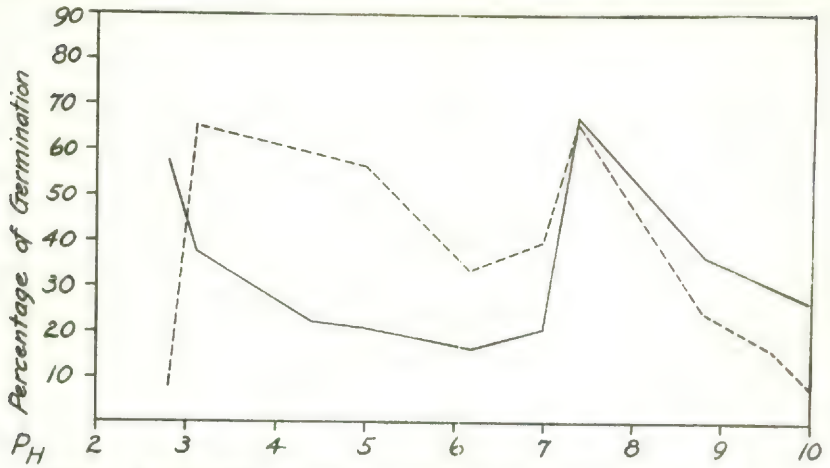

Fig. 4. *Fusarium* sp. Graphic representation of the relation of germination to H ion concentration.

Table IV shows that spores of *Fusarium* sp. are capable of germination over an extremely wide range of reaction. After incubation for 6 hours at 22° C. no germination is evident in the solution made to test P_H 2.8, and the same is true with the culture testing P_H 3.1, in very noticeable contrast with the results yielded by other forms.

Relatively small percentages of germination are obtained at P_H 4.4, and germination gradually increases with decrease in hydrogen ion concentration until a very pronounced maximum is reached at P_H 7.4. After this maximum, the curve declines only to rise suddenly at P_H 10.0+. Upon further incubation, germination progresses rapidly in the extreme acid cultures, that testing P_H 2.8 exhibiting maximum germination on the acid side. From this maximum, germination decreases to the culture possessing the exponent 6.2, rises slightly at P_H 7.0, exhibits the usual maximum at P_H 7.4, and then decreases with decrease in hydrogen ion concentration, the limiting concentration being beyond the culture testing P_H 10.0+.

Examination after incubation for 6 hours at 27° C. shows that no germination is evident at P_H 2.8, a fact noticed with the series incubated at 22° C. Slight germination occurs at P_H 3.1 and increases to the culture exhibiting the value P_H 5.0. Following the fall in germination at P_H 6.2, there occurs a slight rise at P_H 7.0 and the typical maximum at P_H 7.4. After this crest is

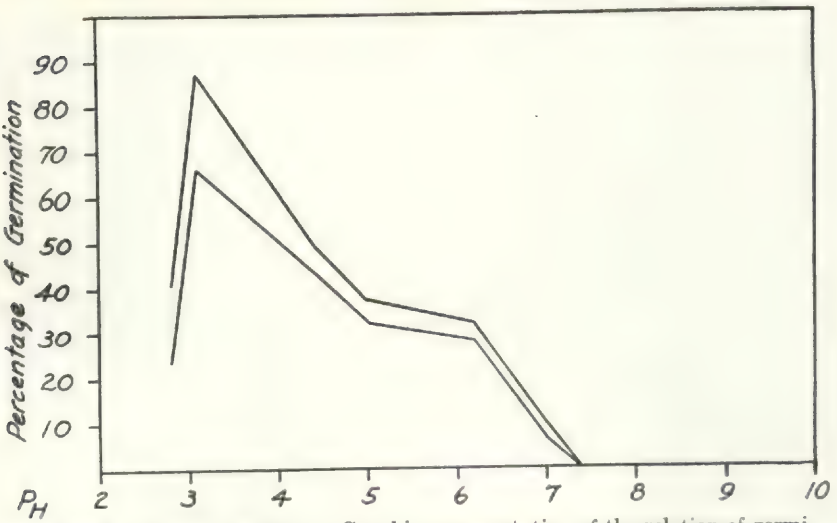

Fig. 5. *Lenzites saepiaria.* Graphic representation of the relation of germination to H ion concentration.

passed, germination decreases rapidly and appears to be totally inhibited at P_H 10.0+.

With an additional incubation of fourteen hours, the order of germination on the acid side shifts considerably, while that on the alkaline side remains practically the same. Slight germination is obtained at P_H 2.8, with the maximum on the acid side occurring at P_H 3.1, and these are the only significant changes. A series was also incubated at 31° C., but on finding that two of the solutions had become contaminated, the data were discarded. In fig. 4 are shown the germination curves for the successful temperatures with this organism.

Very uniform data are obtained with the spores of *Lenzites saepiaria*, as shown by table V.

TABLE V

LENZITES SAEPIARIA. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT 25° C. AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

Hrs.	Hydrogen ion concentration, P_H									
	2.8	3.1	4.4	5.0	6.2	7.0	7.4	8.8	9.6	10.0+
18	23.6	66.3	43.3	32.1	28.3	6.5	0.0	0.0	0.0	0.0
30	40.4	87.4	49.3	37.4	32.2	8.6	0.0	0.0	0.0	0.0

Minimum germination occurs in the culture testing P_H 7.0, and increases with increase of hydrogen ion concentration to the culture with the value P_H 3.1. At P_H 3.1 the maximum is obtained, and with further increase of hydrogen ions there is marked inhibition of germination. The results after incubation of 18 and 30 hours respectively, were very similar.

Due to limited time, it was possible to run only the one series. The curves in fig. 5 represent the germination at 25° C., one curve being constructed from the data after a period of incubation of 18 hours, the other after 30 hours.

Although no controls, as thought of in the usual sense, were run in the experiments reported in this paper, the cultures of each series possessing a hydrogen ion concentration of P_H 7.0 have been regarded as such, it being considered that mannite in doubly distilled water gives a solution with an approximately neutral reaction. Such cultures then contained H and OH ions in equilibrium together with the other ions common to the cultures of the entire series, namely, sodium and phosphorus.

DISCUSSION

It is believed that the results here presented are sufficient materially to change the prevailing view as to the relation of spore germination to acid and alkaline media. Among the forms studied, germination is a process which is strikingly supported by a relatively high hydrogen ion concentration. In certain forms, secondary maxima may occur at approximately the neutral point, but only in one case among those studied, *Fusarium* sp., is the primary maximum near the neutral point or on the alkaline side. It is not necessary, of course, to assume that the hydrogen ion concentration most favorable for germination will also prove most favorable for the continued growth and development of the organism. Moreover, that is a problem outside of the present investigation.

Inasmuch as ordinary nutrient media for pathological and bacteriological work usually exhibit a hydrogen ion concentration approximately neutral or slightly acid, the data obtained from this investigation are further interesting in that they show that successively increasing concentrations of hydrogen ions, from

neutrality, favorably influence germination of the spores of *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, *Fusarium* sp., and *Lenzites saepiaria* up to approximately P_H 3.0. However, with increase of hydrogen ion concentration above this point, the germination quantities abruptly diminish. Some detailed discussion is however needed to compare these results with the work of others.

It has been shown that in the case of *Aspergillus niger*, maximum germination is obtained at P_H 3.1, which expressed in terms of normality is N/1259. At P_H 2.8, or N/631, germination is considerably better than at the neutral point, so that complete inhibition of germination must lie considerably higher than P_H 2.8. Since the foregoing hydrogen ion concentrations have been expressed in terms of normality, it might be well to cite the concentrations of certain acids allowing normal or almost normal development of the spores of *Aspergillus flavus* in beet decoction, as determined by Clark ('99): HCl, N/64; HNO_3, N/64; H_2SO_4, N/128; acetic, N/64; monochloracetic, N/256; dichloracetic, N/128; trichloracetic, N/64; and HCN at N/8192. A mean of the limiting concentrations on the alkaline side for the various temperatures is P_H 8.6, or N/251200, from which it appears that OH ions have the greater toxicity. In beet decoction, Clark found that N/16 KOH injured the spores of *Aspergillus flavus*, while N/8 was fatal; also that N/32 NH_4OH inhibited germination, while N/16 was fatal. He concludes that the hydroxyl group, OH, is rather more toxic to the moulds studied than ionic H. However, as previously shown, the exact concentration of hydrogen ions in his cultures can not be calculated.

Penicillium cyclopium exhibits a relation to hydrogen ion concentration comparable with that of *Aspergillus niger*. Moreover, of all the forms which he studied, Clark found *Penicillium glaucum* the most resistant to acids and alkalis as well as to other poisons, the inhibiting concentrations on the whole being greater than those for *Aspergillus flavus*. Stevens' results indicated that *Penicillium crustaceum* is more resistant to poisons in aqueous solution than any of the other fungi studied by him. Growth occurred in N/50 HCl and H_2SO_4, while N/40 KOH and NaOH caused death. In my study, the rise in the germination

quantities at or about neutral is followed by a general decline. The limiting concentration on the alkaline side is about P_H 10.0+, thus presenting a range of germination greater than that of *Aspergillus niger*.

From my results, *Botrytis cinerea* may be regarded either as very sensitive to an alkaline reaction in mannite solution or else as manifesting a certain dependence upon the stimulating effects of hydrogen ion concentration under such conditions. Not only is germination inhibited at P_H 7.0–7.4, but the maximum, reached at P_H 3.1–2.8, is equivalent to about N/1000 acid. The range of germination in this case is small.

Lenzites saepiaria, like *Botrytis cinerea*, proved very sensitive to an alkaline reaction, and, while the limiting concentration of the former on the acid side is somewhat lower than the latter, the two fungi are similar in behavior. Meacham ('18) obtained inhibition of growth of *Lenzites saepiaria* at about P_H 1.7 in synthetic and malt-extract, and it is of interest to note that he frequently obtained a maximum of growth at about P_H 3.0, which approaches very closely the hydrogen ion concentration of M/5 mannite which affords maximum germination, as reported in this paper.

Of the forms studied, *Fusarium* sp. is the only one that responded favorably to an alkaline medium. Moreover, this form exhibits about the widest range of germination, yet the behavior was variable and discordant results were not infrequent.

Conclusions

Under the conditions described and as far as the experiments have gone, the following conclusions may be drawn:

(1) In a culture solution consisting of M/5 mannite, phosphoric acid, and sodium hydroxide, successively increasing concentrations of hydrogen ions from neutral or approximately neutral to P_H 3.1–2.8 favorably influence germination of the spores of *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, *Fusarium* sp., and *Lenzites saepiaria*.

(2) The range of germination and the magnitude of the germination quantities as influenced by hydrogen ion concentration in the solution mentioned depend upon the organism, germina-

tion being obtained with the following concentrations, inclusive: *Aspergillus niger*, P_H 2.8–8.8; *Penicillium cyclopium*, 2.8–10.0+; *Botrytis cinerea*, 2.8–7.0; *Fusarium* sp., 2.8–10.0+; and *Lenzites saepiaria*, 2.8–7.0.

(3) It is not until a hydrogen ion concentration of P_H 2.8 or above is reached that inhibition of germination of the forms studied is noticed.

(4) *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, and *Lenzites saepiaria* show a maximum of germination in the medium employed at P_H 2.8–3.1; *Fusarium* sp. exhibits a secondary maximum at this concentration.

(5) *Fusarium* sp. gives a pronounced maximum of germination at P_H7.4, and *Penicillium cyclopium* exhibits a minor secondary maximum at P_H 7.0–7.4.

(6) For equal removes from the neutral point, OH ions appear to be relatively more toxic to the spores studied than H ions.

(7) With increase in length of intervals of incubation, the relations of germination to hydrogen ion concentration remain practically the same.

(8) The curves of germination for any organism are practically identical, whether incubated at 22° C., 27° C., or 31° C.

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DIASTASE ACTIVITY IN RELATION TO STAGE OF DEVELOPMENT AND CARBOHYDRATE CONTENT OF THE TUBER OF SOLANUM TUBEROSUM[1]

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The effect upon enzyme activity of various factors, such as temperature, light, and different concentrations of salts, acids, and alkalis, has been studied in considerable detail, and the behavior of the enzymes, with respect to these factors, has thrown much light upon physiological processes. One phase of this question, however, has been touched upon only to a slight extent, that is, the relation of enzyme activity to the various stages of growth of plant organs. For this reason, it was thought that a study of the activity of the enzyme diastase, in relation to tuber growth in *Solanum tuberosum*, might prove profitable. At the same time, it was deemed of interest to follow the changes in starch and sugar content at the different stages, and thus determine whether any correlation exists between diastase activity, growth, and carbohydrate content.

Review of Literature

The fundamental importance of enzymes in the processes of metabolism has resulted in a voluminous literature on the subject, but a survey of this literature reveals only a very few papers which have a direct bearing on the phase of the subject considered in the present instance. These papers are briefly reviewed below. A larger number have a more or less indirect bearing upon the topic here discussed, and some of these will also be considered.

Probably the first observations on the presence of diastase in the potato were made by Payen and Persoz ('33). They found

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of master of arts in the Henry Shaw School of Botany of Washington University.

it to be present in the tubers during growth, and also detected its presence in oats, wheat, maize, and rice during germination. They were the first investigators to prepare the enzyme from the extracts of germinated grain, extracting the latter with water and precipitating the ferment by means of alcohol. To this alcoholic precipitate they applied the term diastase, which has persisted since that time. Subsequent investigation showed diastase to be present not only in tissues in which starch normally occurs as a storage product, but also in some where the reserve materials are stored as sugar. Thus, in 1878, Baranetzky found diastase present in the roots of carrots and turnips, which contain no starch. He also found it in the leaves and stems of several plants and in potato tubers. From this evidence, he suggested that diastase was probably universally present in living cells, and later work has practically confirmed this opinion.

Müller-Thurgau ('82) showed that under certain conditions the amount of cupric-reducing substances in leaves increased at the expense of the starch present. He also found ('85) that exposure of potato tubers to a temperature of 0° C. for a month resulted in an accumulation of sugars, with a corresponding loss of starch. Contrary to popular opinion, it was determined by him that no sugar was formed in potatoes which were actually frozen. He has also found that when potatoes which had become sweet by exposure to low temperatures were placed at a temperature of 8–10° C. the sugar disappeared. Müller-Thurgau considered these phenomena to be due to an enzymic process, which, while more rapid at high temperatures, occurs also at low temperatures. According to his ideas, the lessened respiration at low temperatures, entailing the use of less sugar, together with an inhibition of re-formation of starch from sugar—which re-formation takes place rapidly at high temperatures—allows the sugars to accumulate when potatoes are kept at 0° C.

Brasse ('84) is considered by Brown and Morris ('93) to have been the first to prove conclusively the presence of diastase in leaves. He examined the leaves of the potato, dahlia, beet, tobacco, and some other plants, and measured the rate of the activity of the diastase obtained from extracts by precipitation with alcohol. This product was allowed to act upon starch

paste, the rate of activity being determined by the cupric-reducing power of the solution. He used chloroform to inhibit the growth of microörganisms during the time the enzyme was allowed to act, being one of the first to pay attention to this point.

The relative amounts of different carbohydrates in plants often bear some relation to enzyme activity; therefore, the early work of Hungerbühler ('86), who estimated the carbohydrates at different times in growing potatoes, is of interest here. The following figures give the amounts of reducing sugar, invert sugar, and starch, expressed in percentages of the dry weight, found by him in the tubers at different times:

	June 23	June 30	July 7
Reducing sugars	6.4	.32	.72
Invert sugar		4.50	4.69
Starch	56.7	61.30	66.30

Schulze and Seliwanoff ('88) showed the amount of sucrose in immature potatoes to be especially high. Brown and Morris ('90) determined the amounts of diastase present in barley grains at three stages in their development. The results were as follows:

	Relative diastatic activity
Endosperm half developed	4.4
Endosperm two-thirds developed	7.8
Endosperm completely developed	9.7

These results are in line with the idea that the appearance and increase of the enzyme are related to the formation and nutrition of the embryo.

The same authors studied the germination of barley grains and found the amount of diastase to increase markedly during the process. The diastatic activity of the barley embryos after different periods of germination was found to be as follows:

	Relative diastatic activity
Embryos, dissected from grains which had soaked in water for 24 hours	Trace only
Embryos from same barley, but germinated for 3 days on 5 per cent gelatin. Diastatic activity of embryos plus substrate	.1186
Same, except germinated for 4 days	.1634
Same, except germinated for 6 days	.2432

There has, in general, been agreement among investigators as to the occurrence of diastase in plant tissues, but a notable exception in this respect was Wortmann ('90), who concluded from his experiments that the dissolution of starch in plants is brought about directly by the protoplasm and independently of the diastase present. This conclusion was based on experiments made by him which showed diastase to be absent from most leaves, and to occur in others in such small quantity and in such a state as to exhibit only very feeble activity. Even in those cases where this slight activity was manifested it would not, according to him, account for the transformation of starch which actually takes place.

It is of interest in this connection to note that Schimper ('85) had found that starch-free leaves of *Allium* were much less diastatically active than the leaves of *Tropaeolum*, which contain much starch. The comparatively recent work of Bradley and Kellersberger ('12) in this particular should also be mentioned. They found that the leaves of many different species of plants varied greatly in their diastatic content, some, such as the bayberry, onion, and leek, giving so little reaction as to make the presence of the enzyme doubtful.

Brown and Morris ('93) could not agree with the theory of Wortmann, referred to above, that the protoplasm and not an enzyme is directly responsible for the transformation of starch in leaves, but believed, as did Baranetzky, that diastase is universally present in these organs, and that dissolution of starch is brought about by it. They criticized Wortmann's work, chiefly on the ground that he used the clear filtered leaf extracts in his experiments and did not take into consideration the tannins of the leaves, which they found to interfere greatly with the extraction and activity of the enzyme. The clear filtered leaf extract was found by them to possess much less activity than the same extract before filtering.

This work of Brown and Morris is perhaps the most comprehensive and conclusive that has been carried out upon the presence of diastase in plants. They determined the activity of the enzyme in all cases by the addition of .5 gm. of finely powdered, air-dried leaves to 50 cc. of a 2 per cent starch solution

containing 5 cc. of chloroform per liter as preservative. The digestion was carried on for 48 hours, after which the cupric-reducing power of the solution was determined. Their experiments showed diastase to be present in all leaves examined and that it always possessed a decided activity, sufficient even in the cases of the lowest diastase content to transform more starch than the leaves ever contain at one time. These investigators may therefore be considered to have added further evidence in support of the view of Baranetzky, referred to above, that all living cells contain diastase. Only a few facts remain which render it somewhat doubtful. Duggar and Davis ('14), in this laboratory, were unable to demonstrate the presence of the enzyme in *Fucus*, and Bradley and Kellersberger ('12) were doubtful of its presence in such plants as onions, leeks, and certain mushrooms. Failure to find diastase or invertase in the flesh of ripe apples is also reported by Thatcher ('15).

The comparative tests of diastatic activity made by Brown and Morris, referred to above, showed it to be especially high in leguminous plants, while the members of the *Liliaceae* proved to be poor in the enzyme, corresponding with the small amount of starch they contain. To give an idea as to the relative diastatic activity of the leaves of different species, as determined by them, the following plants are selected from the large number reported:

Plant	Relative diastatic activity
Pisum sativum	240.30
Trifolium pratense	89.66
Solanum tuberosum	8.16
Lycopersicum esculentum	6.57
Allium Cepa	3.76
Hydrocharis Morsus-ranae	.27

Attention is directed to the position of the potato in this table, which indicates a comparatively low diastase content.

When the diastatic activity of a set of half-leaves gathered at one time during the day was compared with that of the corresponding half-leaves picked at another time during the same day, it was found to vary considerably. They consider the figures obtained in the last-mentioned case to indicate that the condi-

tions favorable for starch formation are not favorable for enzyme activity and vice versa, since the enzyme activity was much higher at night than during the day. These findings led Green ('97) to investigate the effect of light upon diastase. He found the activity of the enzyme to be considerably decreased by exposure to sunlight, the deleterious effect varying with the intensity of the illumination.

Brown and Morris also demonstrated that diastase, from certain leaves at least, will hydrolyze solid starch, a point disputed by Wortmann. They concluded, too, that their experiments established almost beyond doubt, that, while protoplasm may exert some influence upon the action of diastase upon starch in the early stages of the action, the dissolution of starch is mainly brought about by the enzyme diastase.

In addition to their investigations of diastase in leaves, these authors also worked upon the carbohydrate content of leaves. They concluded that cane sugar, dextrose, levulose, and maltose are present in leaves, but failed to find any pentoses. The amounts of these sugars were found by them to increase in the light and to decrease in the dark, with the exception of levulose, which in many cases followed a reverse procedure. The increase in levulose in the dark was attributed by them to the inversion of cane sugar.

Finally, these authors believed "starvation" of the cell to induce the dissolution of starch through the formation of diastase, and concluded that of the carbohydrates disappearing from leaves in the dark, maltose and levulose contribute most to the respiratory requirements of the cells. This idea of diastase secretion being induced by starvation of the cell is concurred in by Effront ('02, p. 139), who says: "The secretion [of amylase by barley embryos] is always abundant when the germ is found in poor nutritive condition, and it is checked as soon as an assimilable substance appears."

The work of Meyer ('85) was briefly reviewed by Brown and Morris in connection with their work on the carbohydrates of the leaf. Meyer attempted to ascertain if the leaves of such plants as *Allium Cepa*, which store no starch, contain any other cupric-reducing carbohydrates comparable in amount with the

starch of other leaves. He found that plants which store starch abundantly contain comparatively little soluble reducing and non-reducing carbohydrates in their leaves, while others, such as *Allium Cepa*, *Iris germanica*, and *Gentiana lutea*, which store no starch, accumulate relatively large quantities of soluble reducing substances in their leaves. These reducing substances, according to him, appear to be regulated by the same laws as starch in relation to their appearance and disappearance in light and darkness.

The formation and distribution of diastase in the potato tuber was investigated by Prunet ('92), who observed during germination that diastase activity was greater toward the "seed end" of the potato than elsewhere. This corresponds with the greater development of shoots from that region. He found also a correspondence between the sugars present in the different portions of the tuber and the amount of diastase.

Green ('99) states that in some experiments carried on by him in 1893 he found diastase to be present in the pollen of many plants. The amount usually increased considerably at the onset of germination, and appears, it is stated, "to accompany the tube as the latter elongates, which suggests a formation not only in the grain but in its tube also." As the pollen loses with age the power of germination, it also loses its diastase, which is an indication of the part played by the enzyme in germination. This, Green suggests, is comparable with the influence exerted by diastase in the germination of the potato.

The effect of enzymes upon seed germination was the subject of some study by Waugh ('98). Employing solutions of diastase and some other enzymes, he found that the percentage of germination of old tomato seed was considerably increased, in the case of diastase, where the seeds were soaked in the solution for several hours. Taka-diastase gave somewhat higher, but not as uniformly favorable, results as malt diastase. Trypsin also gave good results in some cases.

The work of Keitt ('11) on the formation of sugars and starch in the sweet-potato led him to believe that in the very immature tubers the sugar might be present either as glucose or sucrose, dependent perhaps upon meteorological conditions. He found

the total sugars to decrease gradually as the plants matured, while the starch apparently increased until the vines were killed by frost, at which time it was at its maximum. Killing of the vines by frost was followed by a breaking down of starch with a tendency toward the formation of sucrose. The following is a typical example of his analyses, in which figures are given for the small immature tubers harvested on August 28, and for successive stages until the potatoes were mature:

Date	Starch	Glucose	Sucrose
Aug. 28	17.88	.61	4.18
Sept. 7	14.91	1.58	1.82
Sept. 18	17.61	1.88	1.91
Sept. 29	19.92	2.30	1.43
Nov. 18	17.82	1.53	2.35

(A freeze occurred on Nov. 6 which killed the vines.)

It has been generally assumed that transformations brought about through the agency of enzymes are reversible; that is, diastase which hydrolyzes starch to maltose also acts as the synthesizing agent in the production of starch. The hydrolytic processes have, of course, received most attention, but have not been exclusively investigated. The synthetic aspect of the problem was attacked by Bradley and Kellersberger ('12). In view of the results obtained, they were unwilling to make any general deductions as to the relation between the diastatic activity of a tissue and its starch-storing function, though their determinations seemed to give considerable support to the view that the enzyme is the synthesizing agent. One reason for their hesitancy in formulating a conclusion was their inability to explain the fact that fresh mushroom tissues, rich in a glycogen-like carbohydrate, when allowed to act upon a soluble starch solution for five days, showed no evidence of diastase activity, while the same tissues ground up with alcohol, washed with ether, and dried, developed considerable activity. They thought this might be explained by the fact that the treatment activated a proenzyme, but if such were the case, they could not understand why in a rapidly growing mushroom in which the glycogen transfer must necessarily be rapid diastase should not be present, if that enzyme is necessary for synthesis.

Some other facts also introduced an element of doubt into the matter. Beets and mangels, which store their carbohydrates as sugar instead of starch, were found to possess highly diastatic leaves, while the roots contained none of the enzyme. No trace of starch could be found in roots of the radish, yet it was one of the most diastatically active tissues studied. Likewise the potato tuber, which is, of course, particularly rich in starch, proved to be poor in diastase. The leaves of different species of plants were found by them to vary greatly in diastase content. The enzyme appeared most abundant in leaves where, to quote them, "starch is never stored permanently, but where it may be found in small amounts during photosynthesis." These statements do not agree very well with that made by Haas and Hill ('17, p. 369), which is as follows: "The amount of diastase is always greater in starch leaves than in sugar leaves, and the same holds for insolated leaves containing much starch, as compared with shaded leaves containing little or no starch."

In studying this problem of carbohydrate synthesis in plants, Bradley and Kellersberger made some determinations of diastatic activity which are of interest in connection with the present problem, and a few of these are given below. The figures have been recalculated on the basis of 100 which represents the greatest activity reported.

Plant	Relative diastatic activity	Amount of starch content
Pea, seeds, medium	100.0	Medium
Pea, seeds, mature	50.0	Abundant
Corn, seeds, young	10.0	Medium
Corn, seeds, medium	6.7	Abundant
Corn, seeds, mature	7.7	Abundant
Corn, cob, young	7.7	Small
Corn, cob, mature	7.7	Small
Corn, husk, young	25.0	None
Corn, husk, mature	8.3	None
Potato, root, medium	2.8	Some dextrin
Potato, tuber, medium	2.9	Very abundant
Potato, leaf, medium	75.0	None*

* Potato leaves generally contain starch, but in this case they were obtained in the early morning before photosynthesis had taken place to any extent.

The diastatic activity of both potato and onion leaves was determined by Brown and Morris ('93) and by Bradley and Kellersberger. Thus it is possible to compare, by means of the values given for onion leaves, the activities of the potato leaves as found in the two cases. Different units, of course, are used to express the enzyme activity in the two cases.

Determination made by	Relative diastatic activity	
	Potato leaves	Onion leaves
Brown and Morris	8.2	3.80
Bradley and Kellersberger	75.0	1.05

It is seen from the above that the relative activity in the case of the potato, as found by Bradley and Kellersberger, was much higher than that found by Brown and Morris. This is doubtless partly due to the fact that the former authors made their determinations by means of the cupric-reducing power of the solutions, while the latter employed the iodine reaction. It illustrates the wide difference that often occurs in the results obtained by different workers along this line. In this case, however, the difference is not one of direction, but of degree only.

The physiological changes taking place in the potato during its rest period have been studied to a considerable degree by Appleman ('11, '16). He found, as did Müller-Thurgau and others, that glucose and sucrose accumulate in potatoes which are stored at 0° C. Diastase activity was also greater at the end of 2 and 4 weeks in the cold-storage potatoes than in those stored at room temperature, but at the end of 6 weeks practically no difference could be detected, as, he explains, this was near the end of the rest period of the variety used in this work. Appleman ('11) says that "the increased diastatic activity is probably due to greater activation of zymogen by free acids which are liberated by the greater permeability of protoplasmic membranes at low temperatures."

It is well known that potatoes will not germinate for several weeks after being harvested, apparently because certain changes known as "after-ripening" must first take place. In attempting to determine the nature of these changes, Appleman ('16) reached the conclusion that carbohydrate transformations in the potato tuber during its rest period must not be considered

after-ripening processes, but simply due to, and dependent upon, changing temperature. He did not find an increase at the time of sprouting in the reducing or total sugars in the case of potatoes which, since harvesting, had been stored under growing conditions; nor was there any difference in the sugar content of the seed and stem ends of the tubers at the beginning of sprouting. The diastase activity, he states, was uniformly greater in the extract from the seed end, but there was no appreciable increase in diastatic activity in either end during the rest period, in spite of the fact that sprouting begins much earlier in the seed end. From this evidence, the conclusion is reached that the "cessation of the rest period is not due to a gradual increase in diastatic activity."

Appleman ('16) found further that sprouting could be brought about at any time during the rest period by removing the skins of the tubers. There was still earlier sprouting in tubers which were cut in half transversely, those buds near the exposed surface starting first. Subdued light and a treatment consisting of wrapping the potatoes with cotton saturated with hydrogen peroxide were both effective in shortening the rest period in new tubers with skins not highly suberized. The author believed the shortening of the rest period in all these cases to be correlated, not with water absorption, but with increased absorption of oxygen.

In his studies of the rest period of plants, Howard ('15) found that when certain agents, such as etherization, desiccation, warm water bath, etc., were used to break the rest period, the diastatic activity of the treated tissues was increased, such increase agreeing in each case with the extent to which the treatment broke the rest period. Corresponding with this increased diastase activity, the amounts of soluble reducing sugars were also found to increase within 24 hours after application of the treatment, provided the treatment was applied during the early winter. Treatments given later in the season were found to have very little effect.

The work of Howard is confirmed to some extent by that of Bonns ('18) in this laboratory. The latter, in studying the effect of etherization upon enzyme activity in corms of *Gladiolus*,

found increased hydrolysis of starch by the enzyme, following treatment with the anaesthetic.

Butler ('13) observed, by means of the coloration produced in slices of tubers by boiling them in Fehling's solution, and also by analyses, the sugar content in potatoes during the rest period and at the time of sprouting. He makes the statement that the accumulation of sugar in the tubers has no particular physiological significance as regards germination. He calls attention to the fact that tubers which germinate at a relatively low temperature contain more sugar than those which germinate at relatively high temperatures, and also that those which are allowed to sprout in the soil have a higher sugar content than those sprouted in the cellar. Certain sprouting potatoes examined by him were found to contain no sugar, though the sprouts themselves contained a considerable amount. The same potatoes, after being stored in an ice chest for 20 days, were found to have accumulated a considerable amount of sugar, while the sugar in the sprouts apparently remained constant. At the same time potatoes taken from storage where the temperature had not fallen below 6° C. showed considerable sugar in both tuber and sprouts. His illustrations show that stored potatoes kept under similar conditions were quite variable as to sugar content, some not containing sugar at all or in the cortex only, others in the medulla only, and still others in both cortex and medulla.

According to some further results obtained by Butler, there is less sugar in the vicinity of sprouting eyes than elsewhere, and by analyses he found the sugar content of the seed ends of sprouting potatoes to be small, as a rule, and to increase toward the stem end. This is at variance with the results of Prunet ('92), who found the greater amount of sugar in the seed end of the tuber, and also with those of Appleman ('16), who found no appreciable difference in the sugar content of the opposite ends of those potatoes examined by him.

Butler believes that the greater metabolic activity at the seed end of the tuber, both during the rest period and at germination, is unfavorable for an accumulation of sugar at that point, and concludes from his data that there is "little if any translocation from remote to budding parts, even in germinating potatoes."

The author states, finally, that since the distribution of sugar in resting and germinating potatoes is not essentially different, its appearance in quantity at the time of germination should be ascribed, partly at least, to metabolic changes induced by other agencies.

Doby and Bodnar ('15) investigated diastatic activity in tubers from healthy potato plants as compared with that in tubers from plants affected with leaf roll. They found the absolute value of the diastase (diastase plus zymogen) to be the same in healthy and diseased potatoes, but, in general, more zymogen was found present in healthy than in diseased tubers. These workers also investigated the presence of zymogen in the extracted juice of potatoes. They observed that the diastatic activity of the juice increased upon standing, which fact they attribute to the rapid conversion, in the extracted juice, of zymogen into amylase. Fresh juice, which was quite active to begin with, did not increase to the same extent as did other juice in which diastase was less active in the beginning. Less zymogen was found to be present in tubers in the middle of the rest period than at other times. It began to increase during the first half of January, and a corresponding increase in diastatic activity was observed at the same time. In the spring only small amounts of zymogen were found, since at that time most of it had been changed into active diastase.

It was believed by these investigators that the diastatic activity of fresh juice depends chiefly upon the stage of development of the tuber, but that it may also be affected by the kind of potato used and by climatic and cultural conditions during growth. The stage of the rest period must also be taken into consideration when determinations are made during that time. Enzyme activity was found to be almost entirely independent of the size of the tubers. This calls attention to the necessity for the consideration of these factors in relative diastatic activity determinations, and emphasizes the importance of a uniform procedure as regards the time that elapses between the different operations necessary in such determinations, as well as the importance of using material, the different stages of which have developed under known and comparable conditions.

One of the last-mentioned authors, Doby ('14), carried out some experiments to determine the effect of the different sugars upon the amylase of potatoes. From the results obtained, he concluded that the action of the enzyme is inhibited by the sugars in the following order, maltose being most effective,—maltose, glucose, fructose, arabinose, galactose, mannose.

He further concluded from his experiments that it is quite probable that the action of amylase in a natural enzyme system is restricted, in the first place by the decomposition products of the substrate, and in the second place by other sugars which may be present.

Since maltose is the final product in the hydrolysis of starch by amylase, it might seem that the activity of the enzyme would soon be checked by the accumulation of this sugar, but Davis, Daish, and Sawyer ('16), in their extensive work upon the formation and translocation of carbohydrates in plants, found that maltose was immediately transformed into hexose by the maltase present, so that there is only a trace of this sugar, in leaves at least, at any one time. Moreover, Norris ('14) states, as a result of experiments upon the factors influencing hydrolysis of glycogen by diastase, that while the mixed products of hydrolysis have a marked retarding influence on the velocity of the reaction, maltose alone has very little effect.

In studying the effect of activators upon the diastase of potatoes, Doby and Bodnar ('15) found, among other things, that the boiled juice exerted a considerable activating influence. Effront ('02) made a similar observation in regard to malt diastase.

Carbohydrate transformations in the sweet-potato resemble to a degree those in *Solanum tuberosum*. Hasselbring and Hawkins ('15, '15a), and Hasselbring ('18), from their studies on this subject, reached the conclusion that, while sweet-potatoes contain only very small amounts of sugar during growth, the transformation of starch into sugar begins immediately after harvesting or upon the killing of the vines by frost. The same changes were found to occur whether the tubers were stored in cellars or remained in the ground. These changes involved, according to the authors, the transformation of starch to reducing sugars

and the subsequent synthesis of cane sugar from these. The formation of sugars was observed to be rapid at a high temperature (30° C.), more gradual at temperatures of 11.7–16.7° C., while lowering the temperature to 4° C. again accelerated the process.

Hasselbring and Hawkins ('15) also found, in the course of further experiments, that the carbohydrate content of the two halves of a potato, which had been split longitudinally, was not the same in all cases. While the analyses of the two halves of a freshly dug tuber agreed closely, differences of from 1 to 16 per cent were found in corresponding halves of tubers which had been kept for a time. These differences were found regardless of the temperature at which the potatoes had been kept. These authors consider their work to confirm and extend the investigations of Keitt, referred to previously.

The extensive work of Davis, Daish, and Sawyer ('16) on the carbohydrates of the mangold leaf, and that of Davis and Sawyer ('16) on the carbohydrates of potato leaves are perhaps the most recent contributions along this line. These investigators not only secured much valuable information regarding distribution of sugars and starch at different hours of the day in the leaves mentioned, but also developed methods for the quantitative determination of these substances in plant material. These methods, while they may, according to Jörgensen and Stiles ('17), be subject to certain errors, are doubtless the most reliable to be found at the present time.

Some of the results obtained in this work were as follows: (1) Starch was found to be entirely absent from mangold leaves except in the early stages of growth, and maltose was never present, either in the leaves, stalks, or midribs, at any time of the day or night. (2) Sucrose was present in the leaf in excess of hexoses in the early stages of growth, but later, when sugar was being stored in the root, the hexoses predominated. The latter sugars were also more abundant than sucrose in the midribs and leaf-stalks. Sucrose was therefore concluded to be the first sugar formed in photosynthesis, being transformed into hexoses for the purpose of translocation. Pentoses were found to form a small proportion of the sugars in the tissues. (3) The

hydrolysis of starch was believed to be effected by a mixture of enzymes, containing maltase in relative excess, so that the transformation of starch to dextrose was complete.

The conclusions in regard to sucrose are in agreement with the views of Brown and Morris ('93). The absence of maltose, however, is contrary to the findings of the latter authors, and is due, according to Davis, Daish, and Sawyer, to the fact that enzyme activity was not checked promptly in the material examined by them. Disagreement also exists in regard to pentoses, Brown and Morris not finding these sugars present.

MATERIALS AND METHODS

Potatoes of the Irish cobbler variety were used in this work. They were grown on a bench in the greenhouse, and, as is usually the case under such conditions, the mature tubers were small, the largest being only 6 cm. in diameter. It was the aim at the outset to make analyses of the tubers for enzyme activity, reducing sugars, sucrose, and starch, beginning when the young potatoes had attained a diameter of 1 cm., and making later determinations corresponding with each increase of 1 cm. in diameter until maturity. This plan has been followed with the exception that it was not possible to obtain sufficient tubers of the first size (1 cm. in diameter) for the determinations of both diastase activity and carbohydrate content, but enough were found for the enzyme activity determinations. Some analyses were also made of seed potatoes.

Upon reaching the proper size the potatoes were harvested, washed free from all particles of dirt, and dried with a towel, after which the analysis of the material was begun without delay. These and all other operations were carried out as quickly as possible, so as to avoid errors which might be due to delay in handling.

This procedure may be objected to by some on the ground that size of the potato tuber is not necessarily correlated with stage of development. In these experiments, however, the first four sizes (1–4 cm. in diameter, inclusive) were all dug at the same time, while the vines were still actively growing, and the 5- and 6-cm. sizes were secured about two weeks later. All

these tubers, except the largest size, which was practically mature, were still in an active, growing condition, and it is believed that they really represented not only different sizes but also different stages of development. This should be especially true in the case of the enzyme activity determinations, as it was necessary in making these to use a rather large number of the tubers to obtain the juice needed, thus providing a composite sample.

Diastase activity.—Two or three methods of obtaining the enzyme were tried before a satisfactory one was found. When the potatoes were sliced and dried by means of alcohol and acetone, according to the method used by Davis ('15), and then finely ground in a mortar, extracted with water, and the enzyme precipitated with 95 per cent alcohol, the diastase so obtained was apparently not active enough to make the method satisfactory.

A method whereby the enzyme was precipitated by alcohol from the pure juice of the potato was then tried. The juice was obtained by quickly grating the tubers, grinding the material in a mortar with carborundum, squeezing the juice out by means of a tourniquet of cheese-cloth, and finally filtering through asbestos and filter-paper. The filtering was done in order to remove the starch present. The enzyme obtained from this juice by precipitation with alcohol apparently possessed no activity whatever, and it was finally decided to use the juice itself, as preliminary tests had shown it to be quite active diastatically. The fact that the juice of the potato has considerable activating influence upon the diastase, as found by Doby ('14), was confirmed, and this no doubt accounts for the inactivity of the alcohol-precipitated enzyme.

In determining diastatic activity, 20 cc. of the fresh juice were added to 100 cc. of a .25 per cent soluble starch solution in a 250-cc. Erlenmeyer flask, 2 cc. of toluol added, and the flasks placed in the incubator and kept at 45° C. for 12 hours. At the end of this time the flasks were placed in boiling water for 10 minutes to kill the enzyme.

At the same time checks were made on the above by substituting 100 cc. of water for the starch in two flasks, incubating one

of these for the same length of time and placing the other in boiling water for 10 minutes to kill the enzyme. The diastase activity determinations therefore included the following in each case: one flask containing juice plus starch, incubated 12 hours; one flask containing juice plus water, incubated 12 hours; one flask containing juice plus water, boiled at once to kill the enzyme. The cupric-reducing power of the solution in the first flask less that of the solution in the third flask is considered to represent the activity of the enzyme.

Some preliminary work seemed to indicate the presence of an activating agent in the potato juice, in the absence of which the enzyme possessed little or no activity. To determine this point, some experiments were carried out according to the following procedure: The enzyme, which was precipitated from about 200 cc. of potato juice by the addition of three volumes of 95 per cent alcohol, was collected on filter-paper and dried, and then dissolved in 100 cc. of water and filtered, after which it was ready for use. Another 200-cc. sample of the juice was boiled, killing the enzyme and precipitating the proteins. This was also filtered before using.

In making the determinations, flasks were prepared as below. The same amounts of diastase solution and boiled juice were used in each instance, water being added to make the volume the same in all cases. Two per cent of toluol was used as a preservative.

One flask containing starch plus enzyme.

One flask containing starch plus enzyme plus boiled juice.
These were incubated for varying periods of 12 to 24 hours at 45° C.

One flask containing enzyme plus boiled juice.
This was boiled at the beginning of the experiment to kill the enzyme.

After incubation the cupric-reducing powers of the solutions were determined and these accepted as an index of the diastatic activity.

Extraction of sugars.—For the sugar determinations, 50 gms. of the green tubers (from the same lot used in the enzyme activity determinations) were weighed out and immediately sliced

into thin slices which were dropped into about 200 cc. of boiling 95 per cent alcohol containing 1 per cent of strong ammonia. After boiling for 10 or 15 minutes, this material was transferred, as nearly quantitatively as possible, to a Soxhlet extraction apparatus and extracted for 18 to 24 hours. After washing out the extraction apparatus with alcohol, this extract, which usually amounted to 400–500 cc., was then evaporated *in vacuo* at a temperature of 35–40° C., leaving finally a volume of 40–50 cc. This extract always contained a certain amount of loose starch, which was removed by centrifuging, after which the extract was made up to 200 cc. with doubly distilled water. When the analyses could not be made at once, 2 per cent toluol was added as a preservative.

This method of extraction and evaporation *in vacuo* is essentially that described by Davis, Daish, and Sawyer ('16) and is quite effective in removing the sugars. The necessity for transferring the material from one vessel to another several times during the process introduces the possibility of error, but with careful handling this is small.

Estimation of reducing sugars.—In making the determinations of reducing sugars, the method described by Shaffer ('14) was employed, with slight modifications. Forty cc. of the sugar solution were used instead of the 10 cc. recommended. This was done on account of the dilution, which made duplicates difficult to obtain when only 10 cc. were used. Five cc. of water and 5 cc. of colloidal iron brought the total to 50 cc., instead of 40 cc. as in the original method used by Shaffer. After removing the iron by centrifuging and filtering, two 15-cc. samples of the filtrate were used for the determinations, each sample corresponding to 12 cc. of the original sugar solution. The dissolved cuprous oxide was titrated with $\frac{N}{20}$ potassium permanganate, and duplicates checked within .1 or .2 cc. of permanganate.

Determination of sucrose.—For the determination of sucrose, the method used was the same up to the point where the two 15-cc. samples were measured out. Then 1.5 gms. (10 per cent) solid citric acid were added to each sample, as recommended by Davis and Daish ('13) for the inversion of sucrose. The samples

plus the citric acid were introduced directly into 50-cc. centrifuge tubes and placed in a boiling water bath for 10 minutes, after which the acid was neutralized to phenolphthalein with normal sodium hydroxide, Fehling's solution added, and the balance of the process completed by the Shaffer method. The sucrose was then determined as glucose by the increase in the cupric-reducing power of the solution, due to inversion of the sucrose.

Starch determinations.—The material from which the sugars had been extracted was used in the starch determinations. This was usually sufficiently dried by standing in the air for a few hours so that it could be ground up finely in a mortar. It was then carefully weighed and exactly $\frac{1}{5}$ of it measured out, made into a paste by adding a small amount of water, and the paste poured into 400 or 500 cc. of boiling distilled water. Then the starch which had been centrifuged out of the sugar solution was suspended in 100 cc. of distilled water, and 20 cc. ($\frac{1}{5}$) pipetted out and added to the other. The whole was then gelatinized by boiling under a reflux condenser for two hours, cooled, and made up to one liter with distilled water, 2 per cent toluol being added to prevent the growth of microörganisms. One-tenth gm. Taka-diastase was then added to 100 cc. of the solution, which was well shaken during sampling, and this was incubated at 45° C. for 24 hours. Under these conditions, according to Davis and Daish ('14), hydrolysis of the starch is complete, it having been broken down into glucose and maltose, which exist in a definite ratio in the solution. These sugars were then determined as glucose. The value found, of course, is not the true starch value, but may be used as a basis for comparison, which is all that is demanded in this work.

RESULTS AND DISCUSSION

Diastase activity.—The results of the diastase activity determinations are given below (table 1). These results are, of course, comparative only. The figures in the first three columns represent the number of cubic centimeters of potassium permanganate used in titrating the dissolved cuprous oxide in one sample, while the relative enzyme activity (the difference between the figures in the first and third columns) is given in the

fourth column. The latter is expressed in terms of 100, which is assumed to be the value of the greatest activity observed.

TABLE I

DIASTASE ACTIVITY IN RELATION TO STAGE OF DEVELOPMENT OF POTATO TUBERS*

Stage of development (diameter in cms.)	No. cc. $\frac{N}{20}$ $KMnO_4$ used in titrating Cu_2O			Relative diastatic activity
	Juice+starch (incubated)	Juice+water (incubated)	Juice+water (boiled)	
1	10.7	Lost	8.0	66
2	13.0	11.4	10.1	71
3	12.8	10.0	8.7	100
4	9.9	7.1	6.5	83
5	6.2	3.5	2.2	98
6	5.7	2.9	1.7	98
Seed potato	11.0	8.4	7.8	78
Seed potato	8.2	6.6	6.5	41
Seed potato	9.6	7.3	7.1	61

* In the growing potatoes, not less than 500 gms. of the tubers were used, in each case, as the source of the juice.

It appears from these figures that advance in stage of development is accompanied by an increase in diastatic activity. The author is inclined to believe that the high value obtained for tubers 3 cm. in diameter is perhaps abnormal and without particular significance.

The diastatic activity of three samples of seed potatoes is also given for purposes of comparison. The first two were taken from the lot that was used for seed, while the third represents a different variety. They had been kept in ordinary storage up to the time the analyses were made. It will be observed that their diastatic activity is considerably lower than that of most of the growing tubers. It is rather surprising that the two samples from the same lot of stored tubers should possess such widely different diastatic activities as 78 and 41.

The second column in table I is included to show the increase in reducing sugars which occurs when the juice alone (diluted

with water) is incubated. If the figures in the third column, which represent the diluted juice boiled at the beginning of the experiment to kill the enzyme, are compared with those in the second, it will be seen that there is a considerable increase in reducing sugars. This is probably due to conversion, by the enzyme, of dextrins in the juice, to reducing sugars. The differences in this respect are less in the case of the seed potatoes, which corresponds with their lower enzyme activity.

The activating effect of boiled potato juice upon the diastase of potatoes has been observed by Doby ('14). The table given below (table II) shows some results obtained by the writer on this point. The figures represent cubic centimeters of potassium permanganate used in titrating the dissolved cuprous oxide. The "enzyme" used is that obtained by precipitation with alcohol, as has already been described.

TABLE II

EFFECT OF BOILED JUICE UPON THE ACTIVITY OF POTATO DIASTASE

Sample	No. cc. $\frac{N}{20}$ $KMnO_4$ used in titrating Cu_2O			Difference due to enzyme in presence of boiled juice
	Enzyme+ starch	Enzyme+ starch+boiled filtered juice	Boiled filtered juice	
1	0	4.1	2.8	1.3
2	0	5.6	4.3	1.3
3	0	2.2	.6	1.6
4	.4	26.0	24.6	1.4
5	.3	5.9	4.9	1.0

From the figures given, it seems that the activity of diastase of potatoes depends upon some substance contained in the juice, but not precipitated by alcohol. It is well known that a number of substances have an activating effect upon diastase, but it has been generally considered that the enzyme when alone would act upon starch, and as far as the author is aware, no one has before presented evidence to show that this is not always the case.

In the first tests made, no activity whatever was observed when the enzyme alone was added to the starch solution, but in

the last two a very small amount of starch was hydrolyzed after 24 hours. While the latter results must be considered positive, the figures are so small as to be almost within the range of experimental error.

Since the activity of diastase is accelerated by small amounts of acids, it was thought that the activating effect of the boiled juice might possibly be due to its hydrogen ion concentration. Accordingly, determinations were made in two cases which gave an H ion concentration of P_H 5.7 and P_H 6.2. This very slight acidity could hardly influence the activity of the enzyme. However, a solution consisting of mono- and dipotassium phosphate having the same P_H value was substituted for the boiled juice in each case, but failed to have any activating effect.

Another point of some interest in regard to diastase activity was incidentally observed in the course of these experiments, that is, the direct effect of the time factor upon diastatic activity of potato juice. Doby and Bodnar ('15) report that when the juice was preserved with toluol and allowed to stand in the dark at 8–10° C. for 24 hours or longer, its diastatic activity increased. As stated, the results reported here were obtained incidentally, without any effort to provide Doby and Bodnar's conditions, and are given for what they are worth (table III). The juice used was preserved with 2 per cent toluol and stood at room temperature in partial light.

TABLE III

EFFECT OF THE TIME FACTOR UPON THE DIASTATIC ACTIVITY OF POTATO JUICE

Sample	Relative diastatic activity	
	Fresh juice	After standing 24 hrs.
1	98	66
2	120	59

These results indicate that under the conditions which obtained in the experiment there is a marked decrease in the diastatic activity of the potato juice on standing 24 hours. This is directly opposed to the conclusions of Doby and Bodnar, who,

however, it must be remembered, kept the juice under different conditions. In view of the results given here, it would seem desirable to repeat the work of the latter authors, employing the conditions under which their results were obtained.

Carbohydrate content of potatoes.—The carbohydrate content of the tubers used in this work is given in table IV. The figures given show the amounts of reducing sugars, sucrose, and starch, all determined as glucose, present in 50 gms. (green weight) of the freshly dug tubers at each stage of growth. No analyses were made of tubers 1 cm. in diameter, due to the fact, as stated before, that while enough of the potatoes of this size were available for the diastase activity determinations, there was not sufficient quantity to make also the sugar and starch determinations.

The results given in table IV show a gradual decrease, with advance in development, in the amount of reducing sugar present, which is accompanied up to a certain point by a corresponding increase in the amount of sucrose. The latter falls off rapidly, however, as the tuber approaches maturity. This is in agreement with the limited data of Hungerbühler ('86), who also found a decrease in the reducing sugars and an increase in invert sugar, with advance in maturity. The results of Schulze and Seliwanoff ('88), who found sucrose to be abundant in the immature tubers, are also confirmed.

TABLE IV

CARBOHYDRATE CONTENT OF POTATO TUBERS AT DIFFERENT STAGES OF GROWTH

Stage of growth (diameter in cms.)	No. gms. carbohydrate, determined as glucose, in 50 gms. green tubers		
	Reducing sugar	Sucrose	Starch
2	.3854	.4217	6.963
3	.3307	.4395	6.039
4	.2388	.6358	6.236
5	.0774	.6194	7.494
6	.0127	.0924	7.632
Seed potato	.1360	.0722	7.065
Seed potato	.1857	.0675	8.650

The results obtained may also be considered to agree with those found by Keitt ('11) for the sweet-potato. The latter found the total sugars in the sweet-potato tuber to decrease toward maturity, and, from the figures given above, the same tendency appears to exist in the Irish potato. In the case of the seed potatoes, the reducing sugars are present in much larger quantity than in the larger sizes of the growing tubers, while the sucrose is lower than the lowest value obtained in the case of the growing potatoes.

It is somewhat difficult to interpret the sugar relations of the potato tuber, as shown by the figures given, but the following is a possible explanation: Sugars in plants are translocated chiefly in the form of the hexoses, glucose and levulose. When photosynthesis and growth are proceeding at a rapid rate in the early stages of development and when diastase activity is not so great as later on, it would be expected that the translocated sugars (reducing sugars), would be present in the potato tuber in comparatively large amounts. Diastase, acting as a synthesizing agent, would gradually convert the glucose into the storage product, starch, but this alone might not suffice to dispose of all the reducing sugars accumulating in the tuber, so the invertase present may also be conceived to act as a synthesizing agent and transform some of it into a temporary storage product, sucrose, which accumulates up to a certain point. Towards maturity, as the translocation of hexoses becomes less rapid and as the glucose which reaches the tuber is more rapidly converted into starch by the more active diastase, less reducing sugar is found present. As the reducing sugar decreases, some of the sucrose is hydrolyzèd to hexose by the invertase, possibly in connection with the general matter of equilibrium relations, and thus a decrease in the sucrose is brought about. Under the influence of the low temperatures which prevail in storage, the starch may be partially hydrolyzed to reducing sugar, some of which may again be built up into sucrose, thus causing an accumulation in the tubers, such as has been observed by a number of investigators.

An examination of the values obtained in the starch determinations shows that the percentage of this carbohydrate increases gradually as the tuber enlarges in size. The relatively high

value found in the case of tubers 2 cm. in diameter does not follow the general trend, and remains unexplained. The two samples of seed potatoes exhibit a rather wide difference in starch content, which, the author believes, is perhaps due to varietal differences or to a difference in the conditions under which the potatoes were kept. The conditions under which the tubers were grown may also have exerted some influence in this respect.

SUMMARY

A review of the literature, bearing directly or indirectly upon the subject of this paper, is presented.

The methods of experimentation are described. They include the determination of diastase activity of potato tubers at various stages of development by adding fresh potato juice to a solution of soluble starch; the extraction and estimation of reducing sugars and sucrose by the comparatively new method of Davis, Daish, and Sawyer; and the estimation of starch by means of Taka-diastase.

The following results were obtained:

Diastase activity and starch content were found to increase with advance in the development of the tubers.

In general, the increase in enzyme activity and starch content of growing potatoes was accompanied by a decrease in the total sugars present.

When the juice of potatoes was preserved with toluol and kept for 24 hours at room temperature, its diastatic activity was found to decrease quite markedly.

Evidence was obtained which indicates that a co-enzyme is necessary in the hydrolysis of starch by potato diastase. The activating agent exists in the juice, and is not destroyed by boiling, nor is it precipitated by alcohol.

It is the opinion of the author that if a number of analyses were made along the lines described and the average of these taken the comprehensive results thus obtained would clear up the points which, in this report, appear somewhat doubtful, and would make it possible to draw definite conclusions.

The writer wishes to acknowledge his indebtedness to Dr.

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THE THELEPHORACEAE OF NORTH AMERICA. XI[1]

TULASNELLA, VELUTICEPS, MYCOBONIA, EPITHELE, and LACHNOCLADIUM

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TULASNELLA

Tulasnella Schroeter, Krypt.-Fl. Schlesien **3**: 397. 1888; Juel, K. Svenska Vet.-Akad. Bihang till Handl. Afd. III. **23**[12]: 21. 1897; Arkiv för Bot. **14**[1]: 8. 1915; Sacc. Syll. Fung. **14**: 234. 1899.—*Prototremella* Patouillard, Jour. de Bot. **2**: 267. 1888.—*Pachysterigma* Johan-Olsen in Brefeld, Untersuch. Myk. **8**: 5. 1889; Engl. & Prantl, Nat. Pflanzenfam. (**1**: 1**): 117. 1898.

Fungi with the aspect of *Corticium* and with simple ovoid to globose basidia but having very large sterigmata, each of which bears a spore.

The organs which have the position of sterigmata—and are so called in the original definition of *Tulasnella* which I have followed—are different from all other sterigmata which I have seen by their spore-like form and greatly constricted connection with the body of the basidium as compared with the diameter of the rest of the sterigma. These organs resemble usual sterigmata in being permanently attached to their basidia. Juel, *loc. cit.*, gives cytological reasons for regarding these organs as basidiospores rather than as sterigmata, but basidiospores not sep-

[1] Issued March 2, 1920.

arable at maturity from the basidia which produce them are not known elsewhere in *Basidiomycetes*, so far as I am aware. Juel's material for cytological study proved to be the hymenium of a *Poria* infested by two species of *Tulasnella*. For the present, it seems less confusing in a taxonomic paper to refer to the spore-shaped organs permanently attached to the basidia in species of *Tulasnella* as sterigmata.

The specimens of *Tulasnella* which I have seen in vegetative condition were slightly colored in such colors as livid pink, dull lavender, and ecru-drab of Ridgway; specimens of all species fade to pale olive-gray in the herbarium. The spores were colored in the mass like the fructifications from which they were obtained in the cases where I secured spore falls on glass from specimens of my collection, but are hyaline under high magnification with the microscope. The fructifications are not adnate, as this term is applied to *Peniophora cinerea*, but merely very thin and tender, for when they are moistened small portions sufficiently large for crushing under a cover glass may be lifted clean from the substratum with the point of a scalpel. Such portions spread out well under the cover glass upon application of pressure and are very satisfactory for observation of the spores and sterigmata.

The species of *Tulasnella* are so similar in aspect that one has to rely upon microscopic details—chiefly of the spores and sterigmata—for recognition of the species. Nineteen species of *Tulasnella* are listed for Europe, but upon such slight differences in dimensions of the spores that it seems probable that the number will be materially reduced when a revision can be made upon the basis of first-hand knowledge of these species.

Tulasnella has been collected in North America in northern United States and Canada only; these gatherings are arranged in three species.

KEY TO THE SPECIES

Spores subglobose, $3\frac{1}{2}$–6×3–$4\ \mu$ ..... *1. T. Eichleriana*
Spores subglobose, 5–$9 \times 4\frac{1}{2}$–$6\ \mu$ ..... *2. T. violea*
Spores more elongated, 10–15×3–$5\ \mu$ ..... *3. T. fusco-violacea*

1. **Tulasnella Eichleriana** Bresadola, Ann. Myc. **1**: 113. 1903; Sacc. Syll. Fung. **17**: 209. 1905; Bourdot & Galzin, Soc. Myc. Fr. Bul. **25**: 32. 1909; Juel, Arkiv för Bot. **14**[1]: 8. 1915.

Fructification effused, thin, pale lilac, finally fading to olive-buff; in structure 20–60 μ thick, composed of interwoven, hyaline hyphae 3 μ in diameter; sterigmata 7–10×3½–4½ μ; spores hyaline, even, 3½–6×3–4 μ.

Fructifications 3–6×1–1½ cm.

On rotting wood and bark of frondose species, rarely on coniferous substrata. Canada, New Hampshire, New York, Idaho, and Washington. July to November.

Fig. 1. *T. Eichleriana.* Young basidium, *a*, beginning formation of sterigmata; older basidium, *b*, having full-grown sterigmata; collapsed basidium, *c*, with spore attached to one sterigma; sterigma, *d*, bearing a spore; spores, *s;* hypha, *h*. × 870.

T. Eichleriana is noteworthy by having the smallest spores and sterigmata which are known in the genus. In these details American collections agree so closely with those of European specimens of *T. Eichleriana* that one can hardly doubt their being this species although authentic specimens have not been at hand for verification.

Specimens examined:

Canada: *J. Macoun, 21.*

Ontario: Ottawa, *J. Macoun, 13.*

New Hampshire: Chocorua, *W. G. Farlow, 1, 4, 6***, and two *unnumbered* specimens (the last three specimens in Mo. Bot. Gard. Herb., 55270, 55276, and 55597), and Nos. *A* and *C* (in Farlow Herb.).

Massachusetts: Sharon, *A. P. D. Piguet, B, E* (in Farlow Herb.).
New York: Ithaca, comm. by G. F. Atkinson, 2817.
Idaho: Priest River, *J. R. Weir, 391* (in Mo. Bot. Gard. Herb., 15657).
Washington: Chehalis *C. J. Humphrey, 6284.*

2. T. violea (Quelet) Bourdot & Galzin, Soc. Myc. Fr. Bul. **25:** 31. 1909.

Hypochnus violeus Quelet, Ass. Fr. Av. Sci. **1882:** 401. 1883.—*Prototremella Tulasnei* Patouillard, Jour. de Bot. **2:** 270. *text f. 1–3.* 1888; Essai Taxon. Hym. 27. *text f. 19.* 1900; Sacc. Syll. Fung. **9:** 236. 1891.—*Tulasnella Tulasnei* (Patouillard) Juel, K. Svenska Vet.-Akad. Bihang till Handl. Afd. III. **23**[12]**:** 21. 1897; Arkiv för Bot. **14**[1]**:** 8. 1915; Sacc. Syll. Fung. **14:** 234. 1899; Bresadola, Ann. Myc. **1:** 114. 1903.—*T. incarnata* Bourdot & Galzin, Soc. Myc. Fr. Bul. **25:** 31. 1909.—An *Corticium incarnatum* var. *pinicolum* Tulasne, Ann. Sci. Nat. Bot. V. **15:** 227. *pl. 10. f. 3–5.* 1872?—Not *Pachysterigmata incarnata* Johan-Olsen in Brefeld, Untersuch. Myk. **8:** 7. *pl. 1. f. 1–2.* 1889.—Not *Corticium roseolum* Karsten, Soc. pro Fauna et Fl. Fenn. Meddel. **16:** 2. 1888.

Illustrations: Patouillard, *loc. cit.*

Type: specimens determined by Quelet in Bourdot Herb. and a fragment in Burt Herb.

Fructification effused, thin, livid pink to dull lavender, fading in the herbarium to olive-buff; in structure 30–70 μ thick, composed of interwoven hyaline hyphae 3 μ in diameter; sterigmata 7–10×5–6 μ, with the main portion nearly spherical; spores subglobose, even, 5–9×4½–6 μ.

Fructifications 1½–6 cm. long, 1–3 cm. broad.

On wood and fallen branches of frondose species, rarely on pine. New England, New York, and Washington. March to November.

This species is distinguished from *T. Eichleriana* by larger spores and sterigmata. The spores are usually about 6 × 5 μ, with a slight point of attachment at the base; the body portion of the sterigma has about the same dimensions as the spores. The fructifications are too thin and tender to permit of large

portions being separated from the substratum, but they are not adnate, for upon moistening the fructification small portions large enough for preparation under a cover glass may be lifted from the substratum with the point of a scalpel.

It seems probable that *Corticium incarnatum* var. *pinicolum* Tul. must have been either the present species or *T. Eichleriana*, on account of the subglobose spores which the Tulasnes figured, although unfortunately without stating spore dimensions or scale of magnification of their figures.

Von Höhnel & Litschauer have published[1] that *Corticium roseolum* Karst. is the same species as *Tulasnella Tulasnei*. I have studied an authentic specimen of *C. roseolum* communicated to me by Karsten; this species is not distinguishable in

Fig. 2. *T. violea*. Young basidium, *y;* young basidium, *a*, forming sterigmata; basidium, *b*, with nearly full-grown sterigmata; old, collapsed basidium, *c*, from whose sterigmata the spores have fallen; spores, *s*. × 870. From specimen determined by Quelet.

coloration and aspect from several sendings of *T. Tulasnei* (= *T. violea*), also on *Betula*, received from Romell and cited below, but it is entirely different in microscopic characters. This specimen of *C. roseolum* agrees well with the description published by Karsten; its spores are hyaline, even, 4–6×3–3½ μ, borne 4 to a basidium on very slender sterigmata of the usual *Corticium* kind; the basidia are simple, cylindric or clavate, 9–10×4–4½ μ; the hyphae are sometimes nodose-septate, and some are incrusted in the region of the substratum. Karsten's publication of *Corticium roseolum* antedates that by Massee and renders unnecessary *Corticium subroseum* Sacc. & Syd. in Sacc. Syll. Fung. **14**: 223. 1899.

[1] K. Akad. Wiss. Wien, Sitzungsber. **115**: 1557. 1906.

Specimens examined:

Sweden: Stockholm, *L. Romell, 125, 141, 142, 143, 149, 150, 184.*

Austria-Hungary: Sonntagberg, *Strasser*, comm. by Bresadola under the name *T. incarnata.*

France: Aveyron, *A. Galzin*, comm. by H. Bourdot, 15423; Allier, *H. Bourdot, 1798*, determined by Quelet, and *3765* under the name *T. incarnata.*

New Hampshire: Chocorua, *W. G. Farlow.*

Vermont: Little Notch, Bristol, *E. A. Burt;* Middlebury, *E. A. Burt;* Chapman's Mill, Middlebury, *E. A. Burt.*

Massachusetts: Magnolia, *W. G. Farlow* (in Farlow Herb.); Sharon, *A. P. D. Piguet*, comm. by W. G. Farlow, N (in Mo. Bot. Gard. Herb., 55002); Sherborn, *H. P. Morse*, comm. by W. G. Farlow; Waltham, *W. G. Farlow* (in Farlow Herb.).

New York: East Galway, *E. A. Burt.*

Washington: Bingen, *W. N. Suksdorf, 906.*

3. T. fusco-violacea Bresadola, Fungi Tridentini **2:** 98. *pl. 210. f. 1.* 1900; Sacc. Syll. Fung. **16:** 203. 1902; Bourdot & Galzin, Soc. Myc. Fr. Bul. **25:** 31. 1909; Juel, Arkiv för Bot. **14[1]:** 8. 1915.

Illustrations: Bresadola, Fungi Tridentini **2:** *pl. 210. f. 1.*

Type: authentic specimen in Burt Herb.

Fructification effused, thin, ecru-drab, fading to pale smoke-gray and pale olive-gray in the herbarium; in structure 40–60 μ thick, composed of hyaline, interwoven hyphae 4–5 μ in diameter; sterigmata 12–15×4½–6 μ; spores hyaline under the microscope, even, 10–15×3–5 μ.

Fig. 3. *T. fusco-violacea.* Basidium, *c*, with fully developed sterigmata; spores, *s;* hypha, *h.* × 870. From authentic specimen from Bresadola. One spore shows a curious projection.

Fructifications 3–5 cm. in diameter.

On bark of *Abies* and sometimes of frondose species. New Hampshire to Pennsylvania. August to December. Rare.

T. fusco-violacea is distinguished from the other species hitherto found in North America by having slender and elongated, rather than subglobose, spores. Bresadola described the color of the fructification as fusco-violaceous when in vegetative condition, drying lilacinus; I have seen dried specimens only, and that from Bresadola is now pale smoke-gray.

Specimens examined:

Sweden: Femsjö, *L. Romell, 418.*

Tyrol: Cavalente, *G. Bresadola.*

New Hampshire: Crawford Notch, *L. O. Overholts, 4883* (in Mo. Bot. Gard. Herb., 56076).

Pennsylvania: Trexlertown, *W. Herbst, 53.*

VELUTICEPS

Veluticeps Cooke emend. Patouillard, Soc. Myc. Fr. Bul. **10:** 78. *pl. 3. f. 1.* 1894; Cooke, Grevillea **8:** 148. 1880 (in part).—*Veluticeps* as a section of *Hymenochaete* Massee, Linn. Soc. Bot. Jour. **27:** 116. 1890; not of Sacc. Syll. Fung. **6:** 600. 1888.

Hymenium velvety with fascicles of colored, flexuous hyphae.

The type species is *Veluticeps Berkeleyi* Cooke, which was published originally as *Hymenochaete veluticeps* Berk. & Curtis.

The fructifications are pileate in the species best known; either dimidiate in our single Cuban species or sessile and attached by the vertex in the species occurring on the opposite side of the world in New South Wales. In both species the fascicles of colored hyphae are 800 μ or more long, about 40–60 μ in diameter, and traverse the whole or a large part of the fructification perpendicular to the surface of the hymenium, beyond which they protrude up to 40–100 μ. The colored hyphae composing the fascicles are about $4\frac{1}{2}$ μ in diameter, cylindric, sometimes granule-incrusted—especially in the deeper portions of the fructification—and are closely crowded together, perhaps 20 or more to a fascicle; they have the character of the colored cystidia, which are scattered between the basidia in the hymenium of *Stereum abietinum, S. glaucescens,* and *S. abnormis,* rather than of the conical, pointed setae characteristic of species of *Hymenochaete.* The genera *Mycobonia* and *Epithele* are closely related to *Veluticeps* by fascicles of hyphae protruding

from the hymenium, but have the fascicles composed of hyaline hyphae.

Veluticeps Berkeleyi Cooke, Grevillea **8**: 149. 1880; Patouillard, Myc. Soc. Fr. Bul. **10**: 77. *pl. 3. f. 1.* 1894.

Hymenochaete veluticeps Berk. & Curtis, Linn. Soc. Bot. Jour. **10**: 333. 1868; Sacc. Syll. Fung. **6**: 600. 1888; Massee, Linn. Soc. Bot. Jour. **27**: 116. 1890.

Illustrations: Myc. Soc. Fr. Bul. **10**: *pl. 3. f. 1.*

Type: in Kew Herb. and in Curtis Herb.

Fructification dimidiate, coriaceous, hard and brittle, on the upper side brown, sulcate-zonate, velutinous, becoming glabrous; hymenium pallid cinnamon, plane thickly studded with protruding fascicles of very dark hyphae; in structure 1–2 mm. thick, composed throughout of colored hyphae arranged in three layers, a broad intermediate layer of longitudinally arranged hyphae which turn upward on the upper side to form the velutinous surface layer and turn downward on the opposite side and terminate in the hymenium; bister-colored hyphal fascicles 40–60 μ in diameter, 800 μ or more long, extend through the under layer of tawny olive subhymenial hyphae and protrude up to 40–60 μ beyond the basidia; spores not found.

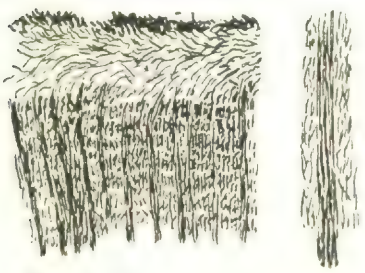

Fig. 4. *V. Berkeleyi.* Section of fructification at left, showing hyphal fascicles, × 19; at right, a single fascicle, × 90.

On logs in woods, often on the under side. May, July. Cuba.

V. Berkeleyi may be recognized by its aspect of a *Hydnum* which upon close examination shows its teeth-like projections on the hymenial side to be really hyphal fascicles not covered by the hymenium. The spores were found to be ovoid and hyaline by Patouillard. Six collections of this species by C. Wright are reported by Berkeley & Curtis in Fungi Cubenses, from which it would seem that the species is common, but I have been able to see no more recent collections from any source. It is possible

that my correspondents have roughly classified their collections of this species as a *Hydnum* and withheld specimens of it.

Specimens examined:

Cuba: *C. Wright, 264* (in Curtis Herb.).

In working over the species of *Aleurodiscus* which have been described, I found that the *Aleurodiscus tabacinus* Cooke should be transferred to *Veluticeps*. Although the species is extra limital and not likely to be found in North America, I now make this transfer and add the following notes on structure:

Veluticeps tabacina (Cooke) Burt, n. comb.

Aleurodiscus tabacinus Cooke, Grevillea **14:** 11. 1885; Handb. Australian Fungi, 193. 1892.—*Corticium tabacinum* (Cooke) Sacc. Syll. Fung. **6:** 607. 1888.

Fructifications pileate, hemispherical or cup-shaped, sessile, apparently attached by the vertex, drying nearly black; in structure 800 μ thick, with a nearly black, crust-like zone on the upper side, from which a broad layer of hyaline hyphae extends to the hymenium and is traversed by brown hyphal fascicles; hymenium drying Verona brown, not covering the protruding fascicles; fascicles about 6 to a mm., 50–60 μ in diameter, up to 900 μ long, protruding up to 100 μ beyond the hymenium, composed of flexuous, colored hyphae 3 μ in diameter; basidia simple, 100×9–10 μ, bearing the spores on 4 slender sterigmata; spores hyaline, even, flattened on one side, 16×6 μ.

Fructifications 2–3 mm. in diameter, 1–1½ mm. thick.

On wood. New South Wales.

V. tabacina is distinct from *V. Berkeleyi* by attachment of its pileus by the center, and by its hyaline substance and subhymenial tissue; when a fertile specimen of *V. Berkeleyi* is available, a difference in spores may perhaps be found.

Specimens examined:

Australia: New South Wales, comm. by G. Massee (in N. Y. Bot. Gard. Herb.).

MYCOBONIA

Mycobonia Patouillard, Myc. Soc. Fr. Bul. **10:** 76. 1894 (with diagnosis under *Bonia* Patouillard, Myc. Soc. Fr. Bul. **8:**

48. 1892, but not *Bonia* Balansa).—*Grandinioides* Banker, Torr. Bot. Club Mem. **12**: 179. 1906.

Thelephoraceous fungi having the hymenium bristling with short cylindric fascicles of hyaline hyphae which arise from the subhymenial tissue.

The type species is *Mycobonia flava*.

Patouillard intended at first that this genus should include both resupinate and pileate species, but he soon transferred the known resupinate species to *Heterochaete* on account of the longitudinally septate basidia. A few years later he introduced *Epithele* in connection with resupinate species, having hyphal fascicles like those of *Mycobonia flava*.

Key to the Species

Fructification sessile ..... *1. M. flava*
Fructification stipitate ..... *2. M. brunneoleuca*

1. Mycobonia flava (Swartz) Patouillard, Myc. Soc. Fr. Bul. **10**: 76. *pl. 3. f. 2.* 1894; *Ibid.* **16**: 180. 1900.

Hydnum flavum Swartz ex Berkeley, Ann. & Mag. Nat. Hist. 1. **10**: 380. *pl. 10. f. 8.* 1842; Linn. Soc. Bot. Jour. **10**: 324. 1868; Sacc. Syll. Fung. **6**: 456. 1888.—*Peziza flava* Swartz, Prodr. 150. 1788; Fl. Ind. Oc. **3**: 1939. 1806.—*Bonia flava* (Berk.) Patouillard in Engl. & Prantl, Nat. Pflanzenfam. (**1.** 1**): 123. *text f. 68G–H.* 1898.—*Grandinioides flavum* (Swartz) Banker, Torr. Bot. Club Mem. **12**: 179. 1906.

Illustrations: Ann. & Mag. Nat. Hist. 1. **10**: *pl. 10. f. 8;* Myc. Soc. Fr. Bul. **10**: *pl. 3. f. 2;* Engl. & Prantl, Nat. Pflanzenfam. (**1.** 1**): *text f. 68 G–H.*

Type: in British Mus. Herb. according to Berkeley, *loc. cit.*

Fructification coriaceous, convex, somewhat orbicular to reniform, sessile, attached by a point on one side, even, glabrous, drying ochraceous buff to cinnamon; hymenium ochraceous buff, with numerous short hyphal fascicles suggesting the teeth of a *Hydnum;* fascicles cylindric, 5–6 to a mm., 60–120×40–60 μ, composed of hyaline or subhyaline hyphae; basidia simple, clavate, 30×6–7½ μ; spores hyaline, even, 10–16×6 μ, not seen attached to the basidia.

Fructifications 1–3 cm. long, 1½–3 cm. broad.

On fallen branches and old logs. Florida, Louisiana, Jamaica, West Indies, and Venezuela. August to November.

When examined by the naked eye or with a magnifying glass, *M. flava* is not distinguishable from a *Hydnum*, but when sections are examined with the compound microscope, the hymenium is found to be a plane surface pierced here and there by the protruding fascicles of hyphae. The spore dimensions are those of spores which were on the surface of the hymenium. A specimen in the collection from Florida has a stem 1 mm. long, but the spores are $13 \times 6\frac{1}{2}$ μ and other characters such that I refer the collection to *M. flava*.

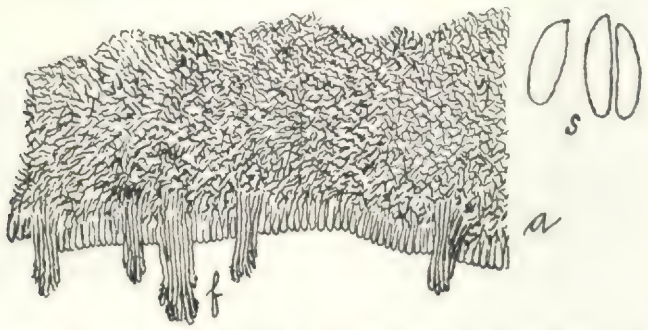

Fig. 5. *M. flava*. Section of fructification, *a*, showing hyphal fascicles, *f*, × 90; spores, *s*, × 870.

Specimens examined:

Florida: Cocoanut Grove, *R. Thaxter* (in Mo. Bot. Gard. Herb., 43985).

Louisiana: St. Martinville, *A. B. Langlois*.

Cuba: *C. Wright* (in Curtis Herb.); Guantonamo (in Weir Herb., 10849); Pinar del Rio San Diego de los Banõs, *N. L. Britton, F. S. Earle & C. S. Gager, 6823* (in N. Y. Bot. Gard. Herb., Burt Herb., and Mo. Bot. Gard. Herb., 56075); Puerto Principe, *F. S. Earle, 312*.

2. M. brunneoleuca (Berk. & Curtis) Patouillard, Myc. Soc. Fr. Bul. **16:** 181. 1900; Duss, Fl. Crypt. Antilles Fr. 233. 1903.

Hydnum brunneoleucum Berk. & Curtis, Linn. Soc. Trans. **22:** 129. 1857; Linn. Soc. Bot. Jour. **10:** 325. 1868; Sacc. Syll.

Fung. **6**: 457. 1888.—*Grandinioides flavum* (Swartz) Banker, Torr. Bot. Club Mem. **12**: 179. 1906 (in part).

Type: in Kew Herb. and Curtis Herb.

Pileus helmet-shaped to flabelliform, vaulted, thin, yellowish brown, slightly streaked behind, glabrous; stem very short, brownish; hymenium whitish, sprinkled with many scattered strong bristles.

Pileus 3½–4 cm. long, nearly as broad.

On dead wood. Martinique and Venezuela.

Patouillard has noted in the place cited that the pileus may attain a diameter of 15 cm., and that the stem is short, thick, and black at the base. Banker includes *M. brunneoleuca* in *M. flava* as a poorly developed form.

I have examined no specimens of *M. brunneoleuca*. The description of the species is that given by Berkeley & Curtis.

EPITHELE

Epithele (as a section of *Hypochnus*) Patouillard, Myc. Soc. Fr. Bul. **15**: 202. 1899.—*Epithele* Patouillard, Essai Taxon. Hym. 59. 1900; Duss, Fl. Crypt. Antilles Fr. 226. 1903; v. Höhn. & Litsch. K. Akad. Wiss. Wien Sitzungsber. **115**: 1595. 1906; Bourdot & Galzin, Soc. Myc. Fr. Bul. **27**: 264. 1911.

Resupinate thelephoraceous fungi lacking an intermediate layer and having the hymenium bristling with short cylindric fascicles of hyaline hyphae which arise from the subhymenial tissue.

The type species is *Epithele Dussii*.

The four species of *Epithele*, known at present, are very thin and delicate in structure and constitute a natural group which is not connected with *Mycobonia* by thick resupinate species with either an intermediate layer or with a doubtful intermediate layer—doubtful merely because the hyphae are interwoven rather than arranged longitudinally in the region of the intermediate layer. *Epithele Typhae* (Pers.) Pat. is a frequent species in Europe on dead leaf bases of *Typha;* if present in the United States, it may have been regarded as one of the *Hydnaceae* on account of the hyphal fascicles in the hymenium.

Key to the Species

Fructification elliptical, white; spores 6–7×2½–3 μ; on tree fern.....*1. E. Dussii*
Fructification interruptedly effused, sulphur-yellow; spores 9–12×7–9 μ; on palmetto.....*2. E. sulphurea*

1. Epithele Dussii Patouillard, Essai Taxon. Hym. 59. 1900; Duss, Fl. Crypt. Antilles Fr. 226. 1903.

Hypochnus Dussii Patouillard, Myc. Soc. Fr. Bul. **15:** 202. 1899; Sacc. Syll. Fung. **16:** 197. 1902.—*Peniophora Dussii* (Patouillard) v. Höhn. & Litsch. K. Akad. Wiss. Wien Sitzungsber. **116:** 749. *text f. 2.* 1907.

Fructification resupinate, very thin, strongly adhering, forming a coating well defined, white or whitish, 3–15×3–4 mm.; fascicles very numerous, erect, white, 20–25 μ in diameter, protruding up to 100 μ, composed of hyphae; basidia 2- or 4-spored, 13×6 μ; spores hyaline, even, attenuated towards the apex, 6–7×2½–3 μ; layer between hymenium and substratum about 20 μ thick.

On dead trunks of tree ferns. Guadeloupe and Venezuela.

The type, which I have not seen, was collected on the dead trunk of *Alsophila aspera.* The collection from Venezuela, cited below, although lacking spores, has the characteristic hyphal fascicles of *Epithele Dussii* and agrees well with Patouillard's description except in being broadly effused. This specimen is 10 cm. long, 1½ cm. wide, and broken off with the substratum along one side and at both ends; hence the fructifications probably become long and widely effused.

Specimens examined:

Venezuela: Mt. El Val, *A. F. Blakeslee, J2,* comm. by W. G. Farlow (in Mo. Bot. Gard. Herb., 13614).

2. E. sulphurea Burt, n. sp.

Type: in Farlow Herb. and Mo. Bot. Gard. Herb.

Fructifications resupinate, interruptedly effused, drying pale sulphur-yellow to marguerite-yellow; in structure 300 μ thick, composed of loosely interwoven, thick-walled, hyaline hyphae 2–3 μ in diameter; fascicles about 9 to a mm., 15–30 μ in diameter, protruding up to 100 μ, composed of hyaline hyphae; basidia

simple, 8–10 μ in diameter, 4-spored; spores hyaline, even, 9–12×7–9 μ.

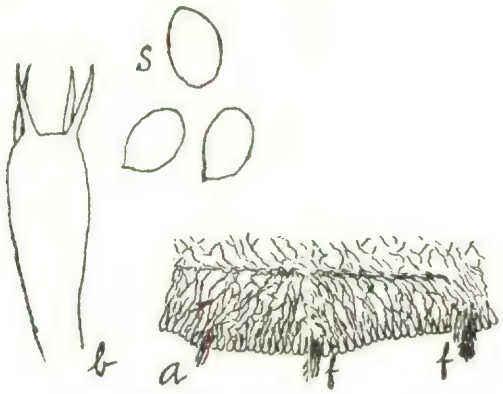

Fig. 6. *E. sulphurea*. Section of fructification, *a*, showing hyphal fascicles, *f*, × 19; basidium, *b*, and spores, *s*, × 650.

On palmetto. Florida. Autumn.

E. sulphurea is noteworthy by its greenish yellow color and spores much larger than those of other species of this genus. Collections of this species are likely to be included in *Hydnum* or *Odontia*, unless examination of sectional preparations is made with the microscope to show that teeth covered by the hymenium are not present.

Specimens examined:

Florida: Palm Beach, *R. Thaxter*, *52*, type (in Farlow Herb. and in Mo. Bot. Gard. Herb., 43940).

LACHNOCLADIUM

Lachnocladium Léveillé in d'Orbigny, Dict. Hist. Nat. **8:** 487. 1846; Morgan, Cincinnati Soc. Nat. Hist. Jour. **10:** 192. 1888; Sacc. Syll. Fung. **6:** 738. 1888; Patouillard, Jour. de Bot. **3:** 23. *pl. 1.* 1889; Engl. & Prantl, Nat. Pflanzenfam. (**1:** 1**): 137. 1898.—*Eriocladus* Léveillé, Ann. Sci. Nat. Bot. III. **5:** 158. 1846, but not of Lindley.

Fructifications coriaceous or somewhat coriaceous, branched, tomentose; branches compressed or terete; coralloid fungi growing on wood or on the ground.

This genus was founded upon a group of seven species, of which none was designated as the type species.

The distinctive characters of *Lachnocladium* are coriaceous consistency and more or less hairy covering of fructifications; by these characters the genus is distinguished from *Clavaria*. At the time of publication of *Lachnocladium* under the name *Eriocladus*, as first proposed, Léveillé restricted the Persoonian genus *Merisma* to glabrous, coriaceous, branched species of the *Clavariaceae*. He had *Clavaria* include fleshy species only, *Merisma*, the glabrous coriaceous species, and *Lachnocladium*, tomentose species so tomentose that the branches were tomentose. Mycologists have not accepted *Merisma* as understood by Léveillé; they have transferred to *Pterula* most of the species which Léveillé had in *Merisma*, and have by their usage modified the idea of *Lachnocladium* by publishing as members of this genus many species which do not have their branches tomentose but differ from branched species of *Clavaria* by being coriaceous.

Lachnocladium comprises a series of species parallel with *Clavaria;* some of the species have hyaline spores, others have more or less ochraceous spores, some, even spores, and some, rough-walled to aculeate spores. Species with dark-colored, more or less rough-walled to muricate spores are better referable to *Thelephora*.

Léveillé regarded *Lachnocladium* as one of the *Clavariaceae* and the genus is located there in Saccardo's 'Sylloge Fungorum' and by Hennings in Engler & Prantl's 'Nat. Pflanzenfam.' Berkeley & Curtis arranged the species of *Lachnocladium* between those of *Thelephora* and *Stereum* in their 'Notices of North American Fungi'[1] and 'Fungi Cubenses.'[2] Patouillard includes *Lachnocladium* in his series of *Thelephores*. In North America there are no species connecting, or intermediate between, *Lachnocladium* and *Thelephora*. While I have had no opportunity to study the various exotic species with dark-colored, echinulate spores which have been published as *Lachnocladium*, it seems very probable that the transfer of such species to *Thelephora* near *Thelephora anthocephala* would

[1] Grevillea 1: 161. 1873.

[2] Linn. Soc. Bot. Jour. 10: 330. 1868.

leave the remaining species of *Lachnocladium* clearly in the *Clavariaceae*.

I include *Lachnocladium* for reference by students of the *Thelephoraceae* because some authors have regarded it as a member of the latter family.

Collectors' field notes on whether the species are coriaceous or fleshy at the time of collecting are necessary for sharply separating *Lachnocladium* and *Clavaria*, for it is evident that these characters may not be well shown in the case of dried specimens of some species.

KEY TO THE SPECIES

Spores hyaline 1
Spores more or less ochraceous 4
Spores dark-colored; in Guadeloupe ..... *11. L. guadelupense*
1. Spores ovoid or cylindric 2
1. Spores subglobose 3
2. Spores even, 3–4½×2–2½ μ; radiately branched organs like those of *Asterostroma* present; Cuba to Brazil ..... *1. L. brasiliense*
2. Spores even, 9×6 μ; fructification somewhat cartilaginous; in Cuba ..... *2. L. cartilagineum*
2. Spores even, 6–12×3–3½ μ; fructification dry, 2½–4 cm. high; on rotting leaves, Vermont to Ohio ..... *3. L. Micheneri*
2. Spores even, 12–15×5–6μ; fructification 3–4 cm. high, everywhere clothed with whitish down; in Pennsylvania ..... *4. L. semivestitum*
2. Spores 7–10×2½–4½ μ; fructifications 8 cm. high; on wood; Connecticut ..... *12 L. odoratum*
3. Spores even, 3–3½×2½–3 μ; fructification 2½ cm. high; on the ground, New Jersey and Pennsylvania ..... *5. L. subsimile*
3. Spores even, 3¾–4½ μ in diameter; fructification 4 cm. high; on wood, Cuba ..... *6. L. cervinum*
3. Spores even, 9½×8–9 μ; on the ground, New Hampshire, Massachusetts, and New York ..... *7. L bicolor*
4. Spores even, 7–12×4½–6 μ; fructification velvety, ochraceous-ferruginous, 7–12 cm. high; on rotten wood, South America ..... *8. L. furcellatum*
4. Spores even, 6–7 × 3–3½ μ; fructification drying drab, clothed with a gray down, 8 cm. high; on wood, West Virginia ..... *9. L. erectum*
4. Spores even, 9–10 × 4½–5½ μ; stem 1 cm. in diameter; branch portion 6–7 cm. high, 5–6 cm. broad; North Carolina ..... *10. L. Atkinsonii*

1. Lachnocladium brasiliense Léveillé, Ann. Sci. Nat. Bot. III. **5:** 159. 1846 (*Eriocladus*); Berk. & Curtis, Linn. Soc. Bot. Jour. **10:** 330. 1868; Sacc. Syll. Fung. **6:** 738. 1888; Patouillard, Jour. de Bot. **3:** 26. *pl. 1. f. 5.* 1889. Plate 5, fig. 1.

Illustrations: Patouillard, *loc. cit.*

Type: stated by Léveillé to be in De Candolle Herb.; Patouillard notes a specimen of original locality and collector—Bahia, *Blanchet*—in Museum of Paris Herb.

Fructification very short-stipitate, most highly branched, coriaceous, drying to tawny olive; branches solid, terete, dichotomous, with slender acute tips; spores hyaline, even, 3–4½× 2–2½ μ, borne on simple basidia; underneath the hymenium radiately branched organs like those of *Asterostroma*, pale-colored, with slender, flexuous rays up to 30×3 μ, are abundant

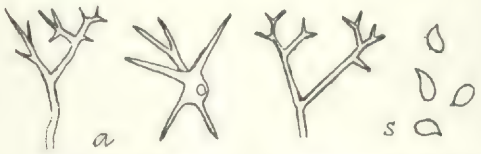

Fig. 7. *L. brasiliense*. Antler-shaped and star-shaped organs, *a*; spores, *s*. × 870.

and form the outer part of the medullary part of the branches and the somewhat spongy outer surface of the fructification where the hymenium is absent.

Fructifications 3–5 cm. high, about 3 cm. in diameter.

On rotting wood. Cuba to Brazil.

L. brasiliense is distinguished by its small, hyaline spores and by the brownish, antler-shaped and star-shaped organs, the latter suggestive of those of *Asterostroma*, which are abundant underneath the hymenium and form the sterile surface elsewhere.

Specimens examined:

Cuba: *C. Wright* (in Curtis Herb., under the name *Thelephora brasiliensis* Lév.); *C. Wright, 831*, under the name *Lachnocladium furcellatum* (in Curtis Herb. and in Mo. Bot. Gard. Herb., 43838).

2. L. cartilagineum Berk. & Curtis, Linn. Soc. Bot. Jour. **10:** 330. 1868; Sacc. Syll. Fung. **6:** 739. 1888; Patouillard, Jour. de Bot. **3:** 26. *pl. 1. f. 4.* 1889. Plate 5, fig. 2.

Illustrations: Patouillard, *loc. cit.*

Type: in Kew Herb. and Curtis Herb.

Fructifications somewhat cartilaginous, erect, drying honey-yellow to olive-brown, densely and repeatedly branched above; branches cylindric, very sharp-pointed; stem slender, cylindric, strigose-hairy at the base; spores hyaline, even, 9×6 μ, slightly flattened on one side, apiculate.

Fig. 8. *L. cartilagineum.* Spores, × 870.

Fructifications 4 cm. high, 1–2½ cm. in diameter; stem 1½–2 cm. long, 1½–2 mm. in diameter.

On the ground. October. Cuba.

Patouillard has noted the spores of this species as ochraceous and a little smaller than I find them. The spores are very abundant in preparations from the type specimen, but the basidia are not well enough preserved to demonstrate whether simple or longitudinally cruciately septate.

Specimens examined:

Cuba: *C. Wright, 204*, type (in Curtis Herb.).

3. L. Micheneri Berk. & Curtis, Grevillea **1**: 161. 1873; Morgan, Cincinnati Soc. Nat. Hist. Jour. **10**: 192. 1888; Sacc. Syll. Fung. **6**: 739. 1888; Hard, Mushrooms, 476. *text f. 401.* 1908. Plate 5, fig. 3.

Clavaria fragrans Ell. & Ev. N. Am. Fungi, 2023. 1888. See Cooke, Grevillea **17**: 59. 1889.—An *Lachnocladium odoratum* Atkinson, Ann. Myc. **6**: 58; 1908?

Illustrations: Hard, Mushrooms, *text f. 401.*

Type: in Kew Herb. and Curtis Herb.

Fructifications gregarious, coriaceous, dry, repeatedly forked and branched and drying drab-gray above; stem cylindric, light buff, tomentose below, arising singly or in a few individuals from more or less effused, mycelial patches on decaying leaves; smaller branches filiform, flexuous, with paler tips; irregular, tomentose patches at various places on main trunk, branches, or axils of branches where hymenium has failed to develop; hymenium glabrous, no cystidia nor hairs present; spores hyaline, even, 6–12 ×3–3½ μ.

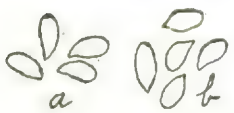

Fig. 9. *L. Micheneri.* Spores, × 87; *a*, from type; *b*, from Burt coll.

Fructifications 2½–4 cm. high, 1–1½ cm. broad; main stem 2–3 mm. in diameter.

On rotting leaves in groves. Canada to New Jersey and westward to Missouri.

This species forms an orbicular, villose or mycelial patch on the surface of leaves—very often beech leaves—and from these patches arise one or two stems, which are tomentose below. In the field notes of this species I have the record, "bitter to taste," but the dried specimens are not bitter now.

Specimens examined:

Exsiccati: Ell. & Ev., N. Am. Fungi, 2023, type distribution of *Clavaria fragrans;* Ell. & Ev., Fungi Col., 1022.

Canada: Ontario, London, *J. Dearness*, in Ell. & Ev., Fungi Col., 1022.

Vermont: Newfane, *C. D. Howe;* Sudbury, *E. A. Burt.*

New York: Snyders, *C. H. Peck* (in N. Y. State Mus. Herb. and in Mo. Bot. Gard. Herb., 56113).

New Jersey: Newfield, *J. B. Ellis*, in Ell. & Ev., N. Am. Fungi, 2023.

Pennsylvania: *E. Michener, 479*, type (in Curtis Herb., 3534); Bethlehem, *Schweinitz*, the *Clavaria crispula* and *C. byssiseda* of Schweinitz, Syn. N. Am. Fungi, 1024 and 1034 respectively (in Herb. Schweinitz).

Ohio: *C. G. Lloyd, 3817* (in Lloyd Herb., Burt Herb., Farlow Herb., and Mo. Bot. Gard. Herb., 44653); Oxford, *L. O. Overholts, 1487* (in Overholts Herb.).

Missouri: Wickes, *E. A. Burt* (in Mo. Bot. Gard. Herb., 43813.)

4. L. semivestitum Berk. & Curtis, Grevillea **1**: 161. 1873; Morgan, Cincinnati Soc. Nat. Hist. Jour. **10**: 192. 1888; Sacc. Syll. Fung. **6**: 739. 1888. Plate 5, fig. 4.

Type: in Kew Herb. and Curtis Herb.

Fructifications coriaceous, erect, repeatedly furcate-branched, the branches terete, rather straight, rising rather close together, everywhere clothed with whitish down except on the final branchlets, drying between light brownish olive and buffy brown; spores of the type hyaline, even, 12–15×5–6 μ.

Fructifications 3–4 cm. high, about 1 cm. in diameter across branches.

On the ground. Pennsylvania.

The fructifications of *L. semivestitum* probably occur solitary or gregarious on the ground. Distinguishing characters are slender, erect habit of growth, appressed branches, and large, hyaline, even spores. In the dried specimen the branches are pruinose rather than hairy. Cooke referred to *L. semivestitum* the specimens distributed by Ell. & Ev., N. Am. Fungi, 2024, under the name *Clavaria velutina* Ell. & Ev. without description, and Ellis & Everhart distributed in Fungi Col., 808, under the name *L. semivestitum* specimens growing on rotten wood in West Virginia, but neither of these distributions can be *L. semivestitum*, for their spores are much too small.

Fig. 10. *L. semivestitum*. Spores, × 870; from type.

Specimens examined:

Pennsylvania: *E. Michener, 1184*, type (in Curtis Herb., 4260).

5. L. subsimile Berk. Grevillea **1**: 161. 1873; Sacc. Syll. Fung. **6**: 739. 1888. Plate 5, fig. 5.

Type: in Kew Herb. and Curtis Herb.

Fructifications coriaceous, slender, delicately and repeatedly dichotomously branched, minutely tomentose except on the branchlets, drying between light brownish olive and buffy brown; spores hyaline, even, 3–3½×2½–3 μ.

Fig. 11. *L. subsimile*. Spores, × 870; *a*, from type; *b*, from Michener specimen in Mo. Bot. Gard. Herb.

Fructification 2½ cm. high, ½ cm. in diameter.

On ground in woods. New Jersey and Pennsylvania. September.

L. subsimile in its dried condition has coloration and general aspect very like *L. semivestitum* but the branches of the former curve rather more apart at the axils and are not as closely appressed above. Only three spores were found in a preparation from the specimen in Curtis Herb., which may be rather immature; these spores are very small in comparison with those of *L. semivestitum*. The specimen distributed in Ell. & Ev., N. Am. Fungi, 2024, under the name *Clavaria velutina* E. & E., without description, and the collection from Pennsylvania, both

of which are cited below as *L. subsimile*, have their spores somewhat rough and may be specifically distinct from this species. Nevertheless I am inclined to regard both collections as the fully mature *L. subsimile*. The type of *L. subsimile* was published as Curtis Herb. No. 4600, which appears to be an error for 4690, the number borne by the specimen to which other data point as the specimen referred to by the description. Ellis notes for his distribution, "Milk white when fresh. Spores white."

Specimens examined:

Exsiccati: Ell. & Ev., N. Am. Fungi, 2024, under the name *Clavaria velutina*.

New Jersey: *Laning, 49*, probable type (in Curtis Herb., 4690); Newfield, *J. B. Ellis*, in Ell. & Ev., N. Am. Fungi, 2024.

Pennsylvania: *E. Michener* (in Mo. Bot. Gard. Herb., 56077).

6. L. cervinum (Berk. & Curtis) Patouillard, Jour. de Bot. **3:** 26. 1888. Plate 5, fig. 9.

Clavaria cervina Berk. & Curtis, Linn. Soc. Bot. Jour. **10:** 338. 1868; Sacc. Syll. Fung. **6:** 716. 1888.—*Clavaria pallida* Berk. & Curtis, Linn. Soc. Bot. Jour. **10:** 338. 1868; Sacc. Syll. Fung. **6:** 714. 1888.—*Lachnocladium pallidum* (Berk. & Curtis) Patouillard, Jour. de Bot. **3:** 26. 1888.

Type: in Kew Herb. and Curtis Herb.

Fructifications coriaceous, branched, becoming tawny olive in the herbarium, hairy with hyaline, thin-walled hairs $1\frac{1}{2}$ μ in diameter which protrude 10 μ beyond the basidia and are longer on the stem; branches repeatedly forked, slender, with very acute tips; spores hyaline, even, subglobose, $3\frac{3}{4}$–$4\frac{1}{2}$ μ.

Fig. 12. *L. cervinum*. Spores, × 870.

Fructifications 4 cm. high.

On dead wood. Cuba. July.

The type of *C. pallida* is a little more densely branched than that of *C. cervina*, but the specimens are so similar in other respects that they can hardly be regarded as different species. Patouillard published the spores as pale ochraceous, but I find them hyaline as seen with the microscope.

Specimens examined:

Cuba: *C. Wright, 235*, type (in Curtis Herb.); *C. Wright, 256*, type of *Clavaria pallida* (in Curtis Herb.).

7. L. bicolor (Peck) Burt, n. comb. Plate 5, fig. 6.

Clavaria bicolor Peck, N. Y. State Mus. Bul. **54**: 954. 1902. Not *C. bicolor* Massee, Kew Bul. **1901**: 154. 1901.—*C. Peckii* Sacc. & D. Sacc. in Sacc. Syll. Fung. **17**: 196. 1905.—*C. vestipes* Peck, N. Y. State Mus. Bul. 116: 35. 1907.

Type: in N. Y. State Mus. Herb.

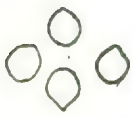

Fig. 13. *L. bicolor.* Spores, × 870.

Fructifications small, 2–2½ cm. high, gregarious; stem slender, 1–2 mm. thick, straight or flexuous, solid, tomentose, pale yellow, divided above into two or more short, orange-colored, compressed branches which are themselves once or twice dichotomously divided; tips acute, concolorous.

Under pine trees. New Hampshire, Massachusetts, and New York. August and September.

The specimens which I have referred to this species are larger in the Massachusetts collection and range from 2½ to 5 cm. high; towards the base the stem is hirsute-tomentose and has dried tawny olive, honey-yellow in the upper portions; the basidia are 45×8 μ, with two sterigmata; and the spores are hyaline, even, subglobose, 9½×8–9 μ. Verification by comparison with the type was overlooked.

Specimens examined:

New Hampshire: Chocorua, *W. G. Farlow* (in Farlow Herb.).
Massachusetts: Coolidge Point, Magnolia, *W. G. Farlow*.

8. L. furcellatum (Fries) Léveillé, as understood by Patouillard, Jour. de Bot. **3**: 26. *pl. 1. f. 3.* 1889; Léveillé, Ann. Sci. Nat. Bot. III. **5**: 159. 1846 (*Eriocladus*); Sacc. Syll. Fung. **6**: 738. 1888; Not of Berk. & Curtis, Linn. Soc. Bot. Jour. **10**: 330. 1868. Plate 5, fig. 7.

Clavaria furcellata Fries, Linnaea **5**: 531. 1830; Epicr. 576. 1838.

Illustrations: Plumier, Filic. Am. *pl. 168. f. L.* 1705; Patouillard, Jour. de Bot. **3**: *pl. 1. f. 3.* 1889.

Fructifications ascending, somewhat ferruginous, with branches solid, repeatedly dichotomous, distant, rather tough, velvety, acuminate.

Fructifications 7–12 cm. high, pallid ferruginous to ochraceous ferruginous. On rotting wood.

The original description, of which the above is a translation, was based upon collections from Guiana by Roxburgh and Brazil by Beyrich, with reference to the same species of a collection from Bourbon Island by Bory, which differed from the South American specimens by decumbent habit, etc.

At the time of publication of *L. furcellatum*, Fries gave only characters sufficient to distinguish this species from an earlier species, *L. tubulosum*, occurring in the same region and having hollow branches. In the course of time several species of

Fig. 14. *L. furcellatum*. Portion of hymenium showing basidia and a hair, *a*; spores, *s*. × 870. From Colombia coll.

South American *Lachnocladium* with solid stems have been recognized, but I have so far failed to find any study upon the original specimens of *Clavaria furcellata* Fries—if these specimens still exist—which gives their microscopical characters and will decide whether *L. furcellatum* as understood by Patouillard or some other *Lachnocladium* with solid branches, is the true *L. furcellatum* (Fries) Lév. The collection from Santa Marta, Colombia, by C. F. Baker, which he distributed under the name *L. brasiliense* upon my determination, I now regard as agreeing more closely with the original description of *L. furcellatum* than

other specimens which I have seen and it has the additional characters published for *L. furcellatum* by Patouillard.

These specimens are tough and certainly coriaceous rather than fleshy, have dried hair-brown below, with final branchlets pinkish buff, everywhere hairy with weak, hyaline hairs 1 μ in diameter, which protrude beyond the basidia except along the tips of the branchlets; spores becoming pale ochraceous, even, 7–12×4½–6 μ, apiculate.

The specimens of *L. furcellatum* of Berk. & Curtis, Fungi Cubenses, are of two species. That collected in Cuba by *C. Wright, 831*, is *L. brasiliense;* the other by *C. Wright, 839*, has small hyaline, even spores 3–4×3 μ but lacks the radiately branched organs characteristic of *L. brasiliense.*

Specimens examined:

Colombia: Bonda, *C. F. Baker, 14*, distributed under the name *Lachnocladium brasiliense.*

9. L. erectum Burt, n. sp. Plate 5, fig. 8.

Type: in Ell. & Ev., Fungi Col., 808, copy in Burt Herb.

Fructifications of the type arise in a cluster of three from a common point, soon repeatedly dichotomously branched, with branches erect, close together, coriaceous, compressed, drying dra), clothed with a gray down whose hyphae are 50–200 μ long; fertile tips of the branches cylindric, flexuous, solid, ½–1 cm. long, bearing the hymenium on all sides; spores very pale yellowish under the microscope, even, 6–7×3–3½ μ.

Fig. 15. *L. erectum.* Spores, × 870.

Cluster of fructifications 8 cm. high, 2½ cm. in diameter in the branched portion; individual stems 1 cm. high, about 2 mm. in diameter; branches about 1 mm. in diameter.

On rotten frondose wood. West Virginia. September.

L. erectum may be distinguished from the other species of its genus in the eastern United States by occurrence on a woody substratum, by its slender, erect habit of growth and appressed branches, by the soft, downy pubescence of weak hyaline hyphae which stand out at right angles from the stem and branches, and by the small, oblong, apparently slightly colored spores.

Specimens examined:

Exsiccati: Ell. & Ev., Fungi Col., 808, type distribution under the name *Lachnocladium semivestitum.*

West Virginia: Nuttallburg, *L. W. Nuttall,* in Ell. & Ev., Fungi Col., 808.

10. L. Atkinsonii Bresadola in Atkinson, Jour. Myc. **8:** 119. 1902; Sacc. Syll. Fung. **17:** 198. 1905.

Type: in Cornell Univ. Herb., 4216.

Fructifications somewhat coriaceous; stem elongated, compressed-canaliculate, pallid, tomentose, 5–6 cm. long, 1 cm. thick, somewhat quadrifid at the apex; branches compressed, sulcate, repeatedly verticillate-, or dichotomo-, divided, tomentose on the sterile side, lurid ochraceous; branchlets somewhat terete, furcate at the apex, straw-yellow; spores hyaline or somewhat straw-colored, even, amygdaliform-oblong or somewhat cylindric, 9–10×4½–5½ μ; basidia clavate.

Dimensions of the branched portion 6–7 cm. high, 5–6 cm. broad. Blowing Rock, North Carolina. August.

A beautiful species approaching the *Clavariae* but included in *Lachnocladium* on account of having the hymenium unilateral and the stem evidently somewhat waxy.

The above is a translation of the original description of this species of which I have seen no specimens.

11. L. guadelupense (Léveillé) Patouillard, Jour. de Bot. **3:** 33. *pl. 1. f. 7.* 1889.

Merisma guadelupense Léveillé, Ann. Sci. Nat. Bot. III. **5:** 157. 1846.—*Pterula guadalupensis* (Léveillé) Sacc. Syll. Fung. **6:** 742. 1888.

Illustration: Patouillard, *loc. cit.*

Type: in Museum of Paris Herb., according to Léveillé.

Fructification with very short stem, coriaceous, branched; branches very thin, elongated, fastigiate, compressed, dichotomous, becoming fuscous; terminal branchlets very short, naked, acute; spores brown, warted, apiculate at base, 12×6 μ.

Stem hardly 1 cm. long.

Guadeloupe.

The above description is a translation of the original description with addition of the spore characters as given by Patouillard. Perhaps the species could be transferred to *Thelephora* with advantage on account of the dark spores; I have seen no specimens. Bresadola includes this species in *Pterula*, in Ann. Myc. **14**: 233. 1916, and gives *Pterula aurantiaca* P. Henn. and *P. squarrosa* P. Henn. as synonyms.

12. L. odoratum Atkinson, Ann. Myc. **6**: 58. 1908; Sacc. Syll. Fung. **21**: 436. 1912.

Type: in Cornell Univ. Herb., 18618.

"Plants 8 cm. high, bases clustered and covered with white mycelium, branches yellowish or grayish, becoming brownish where bruised, branching several times dichotomously, ultimate branches tapering, branched at very tip to make short acute points, branches faintly tinged lemon-yellow, brownish red at very tip, all of larger branches suffused with a reddish tinge, and here and there laterally tomentose, and sterile. Spores transparent, $7–10 \times 3\frac{1}{2}–4\frac{1}{2}$ μ.

"C. U. Herb., No. 18618, growing on very much decayed wood, showing long white cords of mycelium. Connecticut, E. A. White."

The above is the original description. I have seen no authentic specimens but think that they should be compared with *L. Micheneri* and *L. erectum*.

EXCLUDED SPECIES

Pterula setosa Peck, N. Y. State Mus. Rept. **27**: 105. 1875, was transferred to *Lachnocladium* by Sacc. Syll. Fung. **6**: 740. 1888. Patouillard in Jour. de Bot. **3**: 35. 1888, excluded this species from *Lachnocladium*, because its hairiness is due to the elongated sterigmata of the basidia.

(To be continued.)

Explanation of Plate

PLATE 5

The figures of this plate have been reproduced natural size from dried herbarium specimens.

Fig. 1. *Lachnocladium brasiliense*. Collected in Cuba by C. Wright, in Curtis Herb.

Fig. 2. *L. cartilagineum*. From the type in Curtis Herb., collected in Cuba by C. Wright, 204.

Fig. 3. *L. Micheneri*. Collected at Newfane, Vermont, by C. D. Howe.

Fig. 4. *L. semivestitum*. From the type in Curtis Herb., collected in Pennsylvania by E. Michener, 1184.

Fig. 5. *L. subsimile*. From the type in Curtis Herb., collected in New Jersey by Laning, 49.

Fig. 6. *L. bicolor*. Collected at Magnolia, Massachusetts, by W. G. Farlow.

Fig. 7. *L. furcellatum*. Collected at Bonda, Colombia, by C. F. Baker, 14.

Fig. 8. *L. erectum*. From the type in Burt Herb., collected at Nuttallburg, West Virginia, by L. W. Nuttall.

Fig. 9. *L. cervinum*. From the type of *Clavaria pallida* in Curtis Herb., collected in Cuba by C. Wright, 256.

BURT—THELEPHORACEAE OF NORTH AMERICA

1. LACHNOCLADIUM BRASILIENSE.—2. L. CARTILAGINEUM.—3. L. MICHENERI.—4. L. SEMIVESTITUM.—5. L. SUBSIMILE.—6. L. BICOLOR. -7. L. FURCELLATUM.—8. L. ERECTUM.—9. L. CERVINUM.

A SUBTERRANEAN ALGAL FLORA

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That there exists a subterranean algal flora, independent of the terrestrial flora, is a possibility which has seemed so remote that little, if any, attempt has been made to investigate this subject. Many of the earlier writers upon the algae, including Ehrenberg (Mikrogeologie), referred to the algae of the soil, and Gregory,[1] in 1856, discussed somewhat in detail the diatoms obtained from the soil adhering to the roots of dried plants in herbaria.

Robbins,[2] in an account of the algae in some Colorado soils, lists about a dozen blue-greens, one diatom, and two unicellular grass-greens obtained from cultures inoculated with soil. In this case, however, as in all previous accounts, there is no indication that the various forms were not immediately derived from the surface or within a very short distance of the surface of the soil. Robbins removed any loose debris on the surface but the sample consisted of not more than the first three or four inches of earth and included any forms which might have originated terrestrially. These samples, after being thoroughly mixed, were shaken up with distilled water and an amount corresponding to 10 gms. of soil drawn off and distributed over the surface of sterile quartz sand in flasks. Adequate precautions against contamination were observed throughout.

More recently Miss Bristol[3] has reported upon the vitality of algae from old stored soils, but from her account it is obvious

[1] Gregory, W. On the presence of Diatomaceae, Phylolitharia, and sponge spicules in soils which support vegetation. Am. Jour. Sci. and Arts II. **21**: 434–437. 1856.

[2] Robbins, W. W. Algae in some Colorado soils. Colo. Agr. Exp. Sta., Bull. **184**: 24–36. *pl. 1–4.* 1912.

[3] Bristol, B. M. On the retention of vitality by algae from old stored soils. New Phytol. **18**: 92. 1919.

that only samples from the surface were used, and with a single exception any reference in the literature to soil algae may be regarded as having only to do with those forms which grow at or near the surface. Esmarch,[1] however, in a rather extensive paper, attempted to indicate not only the distribution of *Cyanophyceae* upon the surface of various soils but also their occurrence underground. His method was to use Petri dishes about 2 cm. deep, into which 1 cm. of the soil to be studied was introduced. The soil was moistened with sterile water and a piece of filter-paper placed over the surface. The cultures were kept in the greenhouse with diffuse light at a temperature of from 20 to 25° C., and after periods of from two days to two months growth appeared through the filter-paper.

In investigating the distribution of blue-green algae on surface soils an attempt was made to determine whether cultivation influenced their distribution. Accordingly, 4 types of uncultivated soils, including sandy meadow, marshy bog, forest humus, and moist sand, were investigated. On the sandy meadow, which contained traces of humus, but 3 out of 34 samples collected showed the presence of *Cyanophyceae* on the surface. On the marshy bog soil, after a period of three months, none of the 35 cultures showed any blue-green algae, although a few diatoms and grass-green algae were present. Both the forest humus and the moist sand gave good results, so far as indicating the presence of numerous blue-greens on the surface. In cultivated soils, 3 types were used, namely, sandy, clay, and marshy. Of these, 29 out of 45 samples of sandy soil contained *Cyanophyceae*, comprising some 12 different kinds. On the clay soil, 35 out of 37 samples produced blue-green algae with 23 species. On the marshy soil, of the 40 cultures all but 2 showed growth, 22 species being found. While, in general, the above increase indicates that a greater number of blue-greens were found on cultivated than uncultivated soils, the number of samples was so few and taken from such a limited area that no very exact conclusions could be drawn. Any difference in

[1] Esmarch, F. Untersuchungen über die Verbreitung der Cyanophyceen auf und in verscheidenen Böden. Hedwigia 55: 224–273. 1914.

the two types of soil seemed to be determined by the moisture content and the mineral nutrient content.

Coming to the question of the presence of algae underground, Esmarch continued to attempt to correlate their growth with different types of soil. Samples were taken at about the same place as those used for surface cultures, at a depth usually of from 10 to 25 cm. A few, however, extended from 30 to 50 cm. below the surface. All samples were obtained in a manner to prevent surface contamination. The results are grouped according to the kind of soil. In tilled land 13 cultures were made from sandy soil, 12 from clay, and 20 from marshy soil. Only 5 of these contained no blue-green algae and were from places where there were no surface forms. In all, 18 separate species were found and the number decreased as we went deeper into the soil. In the meadow land 23 out of 32 cultures contained blue-greens, with 15 species represented, all these occurring on the surface as well as underground. In moist sand practically all cultures gave results, with 20 different species showing growth. The brown heath and bog soils produced no blue-greens from below the surface.

Esmarch records the occurrence of these subterranean forms as due to the distribution of surface organisms by seepage of surface waters or by being carried down by earth worms and other soil organisms, and, although he was inclined to believe that the blue-greens found by him beneath the surface could grow in the absence of light, he does not regard the work of other investigators on this subject as being altogether conclusive. Furthermore, Esmarch doubts that the blue-green filaments found at considerable depths in the soil have been able to persist there for any length of time. In order to demonstrate this, he prepared cultures in Petri dishes containing 7–8 mm. of soil, on which a piece of filter-paper was placed with certain blue-greens on the surface. The filter-paper was then covered with about 1 cm. of soil, the cultures moistened with distilled water and covered with black paper, the whole being placed in a light-proof case. The temperature was maintained at from 15 to 20° C. After a longer or shorter time, depending upon the

character and mineral content of the soil as well as upon individual differences of the algae themselves, the filaments became discolored, passing from a pale blue-green through a yellowish green to yellow. At first the contents of the cells appeared normal and were apparently in a healthy condition. Later the filaments disintegrated, leaving only spores and heterocysts behind. Cultures which showed practically no normal filaments were removed from the light-proof case, the moistened filter-paper placed on top of the soil, and after about 12 weeks' exposure to daylight again showed blue-green growth. Esmarch regarded this experiment as definitely indicating the impossibility of blue-greens persisting beneath the surface for any length of time, and considered that while the absence of light was a factor, the destructive influence of the soil itself must be taken into consideration.

Aside from this paper of Esmarch's, there appears to be no record of algae growing at considerable depths in the soil, and the investigation here recorded—a preliminary announcement of which was made at the Pittsburgh meeting of the Botanical Society of America, on December 29, 1917—is believed to be the first definite indication that there may exist in the soil, at depths up to 1 m., at least one grass-green alga which is practically always present as a subterranean organism under conditions which preclude its having recently been derived from the surface and accidentally carried down to various depths.

METHODS

The method employed throughout this study was essentially the following:

About 1½ inches of sand was placed in pint milk bottles, to which was added 150 cc. of a culture solution. The bottles were plugged with cotton and sterilized at 8–10 pounds pressure for ½ hour. The culture solution was prepared ½ the strength of the formula of a modified Beyerinck's solution used by Moore,[1] because of the soluble material present in the sand.

[1] Moore, G. T. Methods for growing pure cultures of algae. Jour. Appl. Microsc. 6: 2309–2314. 1903.

The formula undiluted was:

Ammonium nitrate	.5 gm.
Monobasic potassium phosphate	.2 gm.
Magnesium sulphate	.2 gm.
Calcium chloride	.1 gm.
Iron sulphate	Trace
Water	1000 cc.

The bottles were inoculated in duplicate under sterile conditions with about 10 gms. of soil taken from various depths. Every precaution was taken so that the exposed surface was not contaminated with small particles of soil carried down from the upper layers. The spatula by which the samples were taken was sterilized after each inoculation. Checks were run with bottles that were exposed to the air where the inoculations were taken. In order to lessen the amount of evaporation waxed paper covers were placed over the cotton plugs. The sand was slanted in the bottle so that part of it was not submerged, in this way giving various moisture conditions in the culture. The cultures were then placed in cases where they received good light for at least part of the day. The water lost by evaporation was restored from time to time with sterile water.

In order to compare the algal flora of different regions and soil conditions, 10 different series of bottles were inoculated with soil samples from various parts of the Missouri Botanical Garden, 1 from Woods Hole, Massachusetts, and 3 from the vicinity of Santa Ana, California. The varieties of soil examined were heavy clay, loose clay, sand, sandy alkali, sandy gravel and humus. All subterranean cultures were obtained from places where the soil had not been disturbed for at least a number of years. This precaution was necessary in order that the algal growths obtained would represent those typical of subterranean conditions and not merely recent surface infections. At no time did a single check culture show growth, thus eliminating the possibility of algal infection from the air.

SERIES B

INOCULATED OCTOBER 1, 1915. SAMPLE FROM MISSOURI BOTANICAL GARDEN, NORTHWEST CORNER OF LINNEAN HOUSE WALL. FILLED IN TO 45 CM.; BLACK HUMUS TO 20 CM.; BELOW THIS A GRADUAL CHANGE TO PURE CLAY; VERY FINE CLAY AT 37 CM.; DEPTH LIMIT 100 CM.

Ser. B	Depth	Feb. 24, 1916	Mar. 29, 1916	Apr. 11, 1916	June 7, 1916	Aug. 25, 1916	Nov. 14, 1916
B′	Surface			*Protoderma viride*	*P. viride* (motile) *Stichococcus bacillaris*	*P. viride* (motile) *S. bacillaris*	*P. viride* (motile) *S. bacillaris*
B′	Surface		*P. viride*	*P. viride*	*P. viride* (motile)	*P. viride* (motile) *S. bacillaris* *Ulothrix variabilis*	*P. viride* Diatoms
B″	Surface				*P. viride*	*P. viride* (motile) *S. bacillaris* *U. variabilis*	*P. viride*
1	10 cm.		*P. viride* (motile)	*P. viride* *Cladophora* sp. Diatoms	*P. viride* *Cladophora* sp. Diatoms	*P. viride* *Trochiscia?* *U. variabilis* Diatoms	*P. viride* *U. variabilis* Diatoms
1′	10 cm.			*P. viride*	*P. viride*	*P. viride*	*P. viride* Diatoms
2	20 cm.		*P. viride* (motile) Diatoms	*P. viride* (motile) Diatoms	*P. viride* (motile) Diatoms	*P. viride* (motile) Diatoms	*P. viride* (motile) Diatoms
2′	20 cm.						*P. viride* (motile) Diatoms

SERIES B—*Continued*

Ser. B	Depth	Feb. 24, 1916	Mar. 29, 1916	Apr. 11, 1916	June 7, 1916	Aug. 25, 1916	Nov. 14, 1916
3	30 cm.			*P. viride*	*P. viride* (motile)	*P. viride*	*P. viride* *S. bacillaris*
3′	30 cm.						*P. viride* *S. bacillaris*
4	40 cm.	*P. viride*	*P. viride*	*P. viride* (motile)	*P. viride*	*P. viride*	*P. viride* (motile) Diatoms
4′	40 cm.						
5	50 cm.		*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride*
5′	50 cm.		*P. viride*	*P. viride*	*P. viride*	*P. viride* Diatoms	*P. viride* Diatoms
6	60 cm.						Diatoms
6′	60 cm.						Diatoms
7	70 cm.						
7′	70 cm.					*P. viride* Diatoms	*P. viride* Diatoms
7″	70 cm.						*S. bacillaris* Diatoms

8 (80 cm.), 9 (90 cm.), and 10 (100 cm.), no growth.

SERIES C

INOCULATED OCTOBER 1, 1916. SAMPLE FROM MISSOURI BOTANICAL GARDEN, HOLE IN CENTER OF LINNEAN HOUSE. SURFACE STONY AND MOIST; FILLED IN TO 20 CM. WITH CLAY, LIME AND BRICK; 20–40 CM., A MIXTURE OF CLAY AND HUMUS; 40–100 CM., GRADUAL CHANGE FROM CLAY TO VERY FINE CLAY. CULTURES C3 AND C3′ WERE TAKEN IN A TAR-LIKE STRATA; DEPTH LIMIT 100 CM.

Ser. C	Depth	Jan. 14, '16	Mar. 25, '16	Aug. 22, '16	Nov. 10, '18
C	Surface		*Ulothrix variabilis* *Stichococcus bacillaris*	*Protoderma viride* *S. bacillaris* *U. variabilis* *Trochiscia?*	*P. viride* *S. bacillaris* *Trochiscia?* *U. variabilis* (plasmolyzed)
C′	Surface	*P. viride* *U. variabilis* *S. bacillaris*	*P. viride* *U. variabilis* *S. bacillaris*	*P. viride* *S. bacillaris* *U. variabilis* *Trochiscia?*	*P. viride*
C″	Surface		*P. viride*	*P. viride* *S. bacillaris* *U. variabilis* *Trochiscia?*	*P. viride* *U. variabilis*
1	10 cm.			*P. viride*	*P. viride*
1′	10 cm.			*P. viride*	*P. viride*
2	20 cm.			*P. viride*	*P. viride*
2′	20 cm.			*P. viride*	*P. viride*
3	30 cm.			*P. viride*	*P. viride*
3′	30 cm.				*P. viride* *U. variabilis*
4	40 cm.		*P. viride*	*P. viride*	*P. viride*
4′	40 cm.				*P. viride*
5	50 cm.			*P. viride*	*P. viride*
5′	50 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride* *S. bacillaris*
6	60 cm.		*P. viride*	*P. viride*	*P. viride*

SERIES C—*Continued*

Ser. C	Depth	Jan. 14, '16	Mar. 25, '16	Aug. 22, '16	Nov. 10, '18
6′	60 cm.			*P. viride*	*P. viride*
7	70 cm.		*P. viride*	*P. viride*	*P. viride*
7′	70 cm.				*P. viride*
8	80 cm.				*P. viride* Diatoms
8′	80 cm.				*P. viride* (scant growth)
9	90 cm.		*P. viride*	*P. viride* (motile)	*P. viride*
9′	90 cm.				
10	100 cm.				
10′	100 cm.			*P. viride*	*P. viride* (motile)

SERIES D

INOCULATED MAY 10, 1916. SAMPLE FROM MISSOURI BOTANICAL GARDEN, CUT IN NEW EMBANKMENT ALONG ROADSIDE EAST OF NEW PROPAGATING HOUSES. NATURAL FORMATION; BLACK HUMUS TO 20 CM.; CLAY TO VERY FINE CLAY THE REMAINING DEPTH; DEPTH LIMIT 120 CM.

Ser. D	Depth	June 10, 1916	June 17, 1916	Aug. 24, 1916	Mar. 8, 1917	Nov. 22, 1918
D	Surface	*Protoderma viride*	*P. viride* *Cladophora* sp. *Ulothrix variabilis*	*P. viride* *Cladophora* sp. *U. variabilis* Diatoms	*P. viride* *U. variabilis* *Stichococcus bacillaris* Diatoms	*P. viride* *Cladophora* sp. *U. variabilis* *S. bacillaris* Diatoms
D	Surface			*P. viride*	*P. viride*	*P. viride*
1	10 cm.		*P. viride* *Cladophora* sp.	*P. viride*	*P. viride* *U. variabilis*	*P. viride*
1′	10 cm.		*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride*	*P. viride* (plasmolyzed)
2	20 cm.	*P. viride*	*P. viride* *Cladophora* sp.	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	
2′	20 cm.		*P. viride* *Cladophora* sp.	*P. viride*	*P. viride*	*P. viride*
3	30 cm.	*P. viride*	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride*	*P. viride*
3′	30 cm.	*P. viride*	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride*	
4	40 cm.		*P. viride*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	
4′	40 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride* Diatoms	

SERIES D—*Continued*

Ser. D	Depth	June 10, 1916	June 17, 1916	Aug. 24, 1916	Mar. 8, 1917	Nov. 22, 1918
5	50 cm.			*P. viride*	*P. viride*	*P. viride* Diatoms
5′	50 cm.		*P. viride*	*P. viride* Diatoms	*P. viride* Diatoms	
6	60 cm.					
6′	60 cm.			*P. viride*	*P. viride*	*P. viride*
7	70 cm.					
7′	70 cm.			*P. viride*	*P. viride*	*P. viride*
8	80 cm.					
8′	80 cm.			*P. viride* Diatoms	*P. viride* Diatoms	*P. viride* Diatoms
9	90 cm.			*P. viride* Diatoms	*P. viride* Diatoms	*P. viride* Diatoms

100 (100 cm.), 110 (110 cm.), and 120 (120 cm.), no growth.

SERIES E

INOCULATED JUNE 6, 1916. SAMPLE FROM MISSOURI BOTANICAL GARDEN, HOLE IN NORTH AMERICAN TRACT. NATURAL FORMATION; BLACK HUMUS TO 20–30 CM.; BELOW THIS CLAY TO VERY FINE CLAY; DEPTH LIMIT 80 CM.

Ser. E	Depth	Sept. 15, '16	Dec. 16, '16	Mar. 29, '17	Nov. 16, '18
E	Surface		*Protoderma viride*	*P. viride* *Ulothrix variabilis*	*P. viride* *U. variabilis*
E′	Surface			*P. viride* *U. variabilis*	Diatoms
E″	Surface			*P. viride* *U. variabilis* *Cladophora* sp.	*P. viride*
1	10 cm.		*P. viride*	*P. viride*	*P. viride*
1′	10 cm.			*P. viride*	*P. viride*
2	20 cm.		*P. viride*	*P. viride* *U. variabilis*	*P. viride*
2′	20 cm.			*P. viride* *U. variabilis*	
3	30 cm.		*P. viride*	*P. viride*	*P. viride*
3′	30 cm.			*P. viride*	*P. viride*
4	40 cm.			*P. viride*	*P. viride*
4′	40 cm.			*P. viride*	*P. viride*
5	50 cm.			*P. viride*	*P. viride*
5′	50 cm.				
6	60 cm.		*P. viride*	*P. viride*	*P. viride*
6′	60 cm.				
7	70 cm.			*P. viride*	*P. viride*
7′	70 cm.				*P. viride*
8	80 cm.				

SERIES F

INOCULATED JUNE 6, 1916. SAMPLE FROM MISSOURI BOTANICAL GARDEN, HOLE, AT EDGE OF WOODED NORTH AMERICAN TRACT, DUG UNDER TREES. NATURAL FORMATION; BLACK HUMUS TO 20–30 CM.; REMAINING DEPTH CLAY TO VERY FINE CLAY; DEPTH LIMIT 70 CM.

Ser. F	Depth	Aug. 25, 1916	Sept. 15, 1916	Sept. 19, 1916	Mar. 30, 1917	Nov. 19, 1916
F	Surface		*Protoderma viride*	*P. viride* *Ulothrix variabilis*	*P. viride*	*P. viride*
F′	Surface		*P. viride*	*P. viride* *U. variabilis* *Stichococcus bacillaris*	*P. viride* (motile)	*P. viride*
F″	Surface		*P. viride*	*P. viride* *U. variabilis*	*P. viride* (motile)	*P. viride* (motile)
1	10 cm.		*P. viride* *U. variabilis*	*P. viride*	*P. viride* *U. variabilis*	*P. viride*
1′	10 cm.		*P. viride*	*P. viride*	*P. viride*	*P. viride*
2	20 cm.		*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*
2′	20 cm.		*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis*
3	30 cm.		*P. viride*	*P. viride*	*P. viride*	*P. viride*
3′	30 cm.		*P. viride* *Trochiscia?*	*P. viride* Diatoms	*P. viride*	*P. viride*
4	40 cm.		*P. viride*	*P. viride* Diatoms	*P. viride* Diatoms	*P. viride* Diatoms
4′	40 cm.			*P. viride*	*P. viride*	*P. viride*
5	50 cm.		*P. viride*	*P. viride* *U. variabilis*	*P. viride*	*P. viride* *U. variabilis*
5′	50 cm.			*P. viride*		*P. viride* *U. variabilis*

SERIES F—*Continued*

Ser. F	Depth	Aug. 25, 1916	Sept. 15, 1916	Sept. 19, 1916	Mar. 30, 1917	Nov. 19, 1916
6	60 cm.		*P. viride* *Trochiscia?*	*P. viride* *U. variabilis*		*P. viride* *U. variabilis*
6′	60 cm.			*P. viride* *U. variabilis*		*P. viride*
7	70 cm.		*P. viride*	*P. viride*		
7′	70 cm.					

SERIES G

INOCULATED SEPTEMBER 26, 1916. SAMPLE TAKEN FROM MISSOURI BOTANICAL GARDEN, NEWLY EXCAVATED TRENCH PARALLEL WITH LINNEAN HOUSE WALL. PACKED ROAD-BED INTERMIXED WITH TAR TO 10 CM.; NATURAL FORMATION AT 20 CM.; REMAINING DEPTH GRADUALLY GRADING INTO CLAY; DEPTH LIMIT 40 CM.

Ser. G	Depth	Nov. 2, '16	Mar. 12, '17
G	Surface	*Protoderma viride* *Stichococcus bacillaris* *Ulothrix variabilis*	*P. viride* *S. bacillaris* *U. variabilis*
G′	Surface	*P. viride* *Cladophora* sp. *S. bacillaris* *U. variabilis*	*P. viride* *Cladophora* sp. *S. bacillaris* *U. variabilis*
1	10 cm.	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.
1′	10 cm.	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.
2	20 cm.	*P. viride*	*P. viride*
2′	20 cm.	*P. viride*	*P. viride*
3	30 cm.	*P. viride*	*P. viride*
3′	30 cm.	*P. viride*	*P. viride*
4	40 cm.	*P. viride*	*P. viride*
4′	40 cm.	*P. viride*	*P. viride*

SERIES H

INOCULATED OCTOBER 12, 1918. SAMPLE FROM MISSOURI BOTANICAL GARDEN, NEWLY EXCAVATED AREA IN NORTH END NEAR SERVICE SHOPS. CLAY AND HUMUS TO 25 CM.; REMAINING DEPTH CLAY; DEPTH LIMIT 100 CM.

Ser. H	Depth	Nov. 20, '18	Jan. 21, '19	Mar. 21, '19	July 10, '19
H	Surface		*Protoderma viride* *Ulothrix variabilis*	*P. viride* *Nostoc muscorum* *Oscillatoria formosa* *O. anoema* *O. splendida* *Scytonema Hofmanni* *Navicula atemoides* *Nitzschia Kützingiana* *Hantzschia amphioxys*	*P. viride* *N. muscorum* *O. formosa* *O. anoema* *O. splendida* *S. Hofmanni* *N. atemoides* *N. Kützingiana* *H. amphioxys* *Stichococcus bacillaris*
H′	Surface				
1	5 cm.	*P. viride*	*P. viride* *N. muscorum* *O. chlorina* *N. atemoides* *N. Kützingiana*	*P. viride* *N. muscorum* *O. chlorina* *N. atemoides* *N. Kützingiana* *Cladophora* sp.	*P. viride* *N. muscorum* *O. chlorina* *N. atemoides* *N. Kützingiana* *Cladophora* sp.
1′	5 cm.		*P. viride* *N. muscorum* *S. Hofmanni* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*	*P. viride* *N. muscorum* *S. Hofmanni* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*	*P. viride* *N. muscorum* *S. Hofmanni* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*
2	10 cm.	*P. viride*	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana*
2′	10 cm.	*P. viride*	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *Cladophora* sp.	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *Cladophora* sp.	*P. viride* *N. muscorum* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *Cladophora* sp.

SERIES H—*Continued*

Ser. H	Depth	Nov. 20, '18	Jan. 21, '19	Mar. 21, '19	July 10, '19
3	15 cm.	*P. viride*	*P. viride* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *Hantzschia amphioxys*	*P. viride* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *H. amphioxys*	*P. viride* *O. amphibia* *O. subtilissima* *N. atemoides* *N. Kützingiana* *H. amphioxys*
3′	15 cm.	*P. viride* *O. amphibia* *O. subtilissima* *S. Hofmanni* *N. atemoides* *N. Kützingiana* *H. amphioxys*	*P. viride* *O. amphibia* *O. subtilissima* *S. Hofmanni* *N. atemoides* *N. Kützingiana* *H. amphioxys*	*P. viride* *O. amphibia* *O. subtilissima* *N. atemoides* *S. Hofmanni* *N. Kützingiana* *H. amphioxys*	*P. viride* *O. amphibia* *O. subtilissima* *N. atemoides* *S. Hofmanni* *N. Kützingiana* *H. amphioxys*
4	20 cm.	*P. viride*	*P. viride* *U. variabilis*	*P. viride* *U. variabilis* *O. amphibia* *O. chlorina* *O. subtilissima*	*P. viride* *U. variabilis* *O. amphibia* *O. chlorina* *O. subtilissima*
4′	20 cm.	*P. viride*	*P. viride* *U. variabilis* *O. amphibia* *O. chlorina* *O. subtilissima* *N. muscorum* *S. Hofmanni* *N. atemoides* *N. Kützingiana*	*P. viride* *U. variabilis* *O. amphibia* *O. chlorina* *O. subtilissima* *N. muscorum* *S. Hofmanni* *N. atemoides* *N. Kützingiana*	*P. viride* *U. variabilis* *O. amphibia* *O. chlorina* *O. subtilissima* *N. muscorum* *S. Hofmanni* *N. atemoides* *N. Kützingiana*
5	25 cm.		*P. viride*	*P. viride*	*P. viride*
5′	25 cm.				
6	30 cm.		*P. viride* *U. variabilis* *Cladophora* sp. *N. atemoides*	*P. viride* *U. variabilis* *Cladophora* sp. *N. atemoides*	*P. viride* *U. variabilis* *Cladophora* sp. *N. atemoides*
6′	30 cm.	*P. viride*	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*
7	35 cm.	*P. viride*	*P. viride* *N. atemoides*	*P. viride* *U. variabilis* *N. atemoides*	*P. viride* *U. variabilis* *N. atemoides*
7′	35 cm.	*P. viride*	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*

SERIES H—*Continued*

Ser. H	Depth	Nov. 20, '18	Jan. 21, '19	Mar. 21, '19	July 10, '19
8	40 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
8′	40 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
9	45 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
9′	45 cm.	*P. viride*	*P. viride*	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.
10	50 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
10′	50 cm.	*P. viride*	*P. viride* *Trochiscia*?	*P. viride*	*P. viride*
11	55 cm.	*P. viride*	*P. viride* *Trochiscia*?	*P. viride*	*P. viride*
11′	55 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
12	60 cm.		*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.	*P. viride* *Cladophora* sp.
12′	60 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
13	65 cm.	*P. viride*	*P. viride*	*P. viride*	*P. viride*
13′	65 cm.		*P. viride*	*P. viride*	*P. viride*
14	70 cm.				
14′	70 cm.				
15	80 cm.				
15′	80 cm.		*P. viride*	*P. viride*	*P. viride*
16	90 cm.		*P. viride*	*P. viride*	*P. viride*
16′	90 cm.		*P. viride*	*P. viride*	*P. viride*
17	100 cm.		*P. viride*	*P. viride*	*P. viride*
17′	100 cm.		*P. viride*	*P. viride*	*P. viride*

SERIES J

INOCULATED JUNE 27, 1919. SAMPLE FROM MISSOURI BOTANICAL GARDEN, ABOUT 10 CM. FROM THE PLACE IN SERIES H; DEPTH LIMIT 100 CM.

Ser. J	Depth	Sept. 23, '19	Nov. 4, '19
J	Surface	*Protoderma viride* *Nostoc muscorum* *Hantzschia amphioxys*	*P. viride* *N. muscorum* *H. amphioxys*
J′	Surface	*P. viride*	*P. viride*
1	5 cm.	*P. viride*	*P. viride* *Cladophora* sp. *H. amphioxys*
1′	5 cm.	*P. viride*	*P. viride* *Cladophora* sp. *H. amphioxys*
2	10 cm.	*P. viride* *Ulothrix variabilis*	*P. viride* *U. variabilis* *Cladophora* sp.
2′	10 cm.	*P. viride* (motile)	*P. viride* *Navicula atemoides*
3	15 cm.	*P. viride*	*P. viride* *N. atemoides*
3′	15 cm.	*P. viride*	*P. viride* *N. atemoides*
4	20 cm.		
4′	20 cm.		
5	25 cm.	*P. viride*	*P. viride* *N. atemoides*
5′	25 cm.	*P. viride*	*P. viride*
6	30 cm.	*P. viride* (motile)	*P. viride*
6′	30 cm.	*P. viride*	*P. viride*
7	35 cm.		*P. viride*

SERIES J—*Continued*

Ser. J	Depth	Sept. 23, '19	Nov. 4, '19
7′	35 cm.	*P. viride*	*P. viride*
8	40 cm.	*P. viride*	*P. viride*
8′	40 cm.	*P. viride*	*P. viride*
9	45 cm.		
9′	45 cm.		
10	50 cm.	*P. viride*	*P. viride*
10′	50 cm.	*P. viride*	*P. viride*
11	55 cm.	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*
11′	55 cm.	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*
12	60 cm.	*P. viride*	*P. viride* *Oscillatoria amphibia*
12′	60 cm.	*P. viride*	*P. viride*
13	65 cm.	*P. viride*	*P. viride*
13′	65 cm.	*P. viride* (motile)	*P. viride* *Trochiscia?*
14	70 cm.	*P. viride* *H. amphioxys* *N. atemoides*	*P. viride* *H. amphioxys* *N. atemoides* *O. amphibia*
14′	70 cm.	*P. viride* *H. amphioxys* *N. atemoides*	*P. viride* *H. amphioxys* *N. atemoides*
15	75 cm.		*P. viride*
15′	75 cm.		

SERIES J—*Continued*

Ser. J	Depth	Sept. 23, '19	Nov. 4, '19
16	80 cm.	*P. viride* (motile) *Trochiscia?*	*P. viride* (motile) *Trochiscia?*
16′	80 cm.	*P. viride*	*P. viride* (motile)
17	85 cm.	*P. viride*	*P. viride*
17′	85 cm.	*P. viride* *N. atemoides*	*P. viride* *N. atemoides*
18	90 cm.	*P. viride*	*P. viride*
18′	90 cm.	*P. viride*	*P. viride* *H. amphioxys* *N. atemoides*
19	95 cm.	*P. viride* (motile)	*P. viride* *N. atemoides*
19′	95 cm.	*P. viride*	*P. viride* *N. atemoides*
20	100 cm.		*P. viride* *H. amphioxys* *N. atemoides*
20′	100 cm.		*P. viride* *H. amphioxys* *N. atemoides*

Series A, inoculated with very poor soil from the Missouri Botanical Garden, was a preliminary series and not carefully examined, so that no record is tabulated. Cultures were taken at the surface and at 20, 40, and 60 cm. below the surface. *Protoderma viride* was found in all the cultures and *Anabaena* appeared in those taken at a depth of 20 cm.

Series H contained a greater number of blue-green forms than any of the other series, and it seemed desirable to repeat the experiment. Series I was inoculated March 6, and Series J, June 27, 1919, with soil taken within a few centimeters of the area used in Series H. The surface of the embankment was scraped off to expose a clean area. At the end of 3 months in Series I, and 40 days in Series J, no growth was apparent, whereas in Series H growth had been abundant in almost all of the bottles in the latter period of time.

Since Series I showed no growth at the end of 90 days, the cultures were discarded. Growth in Series J first appeared at the end of about 40 days. Even at the end of 3 months these cultures showed less growth and fewer species of algae than those of Series H. However, *Protoderma viride* again appeared throughout. The fact that there was no growth in Series I and that fewer species of algae appeared in Series J may have been due to the surface of the embankment having been exposed during the winter months and the low temperature probably having killed some of the forms originally present in Series H. This exposure probably killed many or all of the vegetative cells of the algae which survived, and thus the delayed growth in Series J might be explained by the persistence of spores which required a longer period of time in which to produce a visible growth.

The cultures of soil from Woods Hole, Mass. and Santa Ana, Calif. were taken in exactly the same manner to a depth of 1 meter as the preceding ones. The soil at Woods Hole was sandy gravel containing several large boulders. The series taken at Santa Ana were especially valuable because of the different soil conditions, one series being taken from very sandy soil and another from sandy alkali soil. The third series was taken

from ordinary garden soil. No tabulated results were kept of these because *Protoderma* appeared in all of the cultures.

From the above tables, it will be seen that there exists a subterranean algal flora independent of the nature of the soil and the locality. A wide variety of algae does not appear in the soils examined but in most cases the variety is as great as at the surface. The absence of a variety of blue-green algae and the constant occurrence of *Protoderma viride* is especially noticeable. The fact that the latter occurs at the greatest depth and in every soil seems to indicate that it is especially adapted to live under subterranean conditions.

The greater number of soil samples studied in this investigation is comparable to those termed uncultivated forest soils by Esmarch. The results in general are also similar in that he found no *Cyanophyceae* on the surface or underground. The soils in all cases were uncultivated, a fact which may account for fewer cultures showing blue-green forms than reported by Robbins and Esmarch. It is possible that the unicellular green alga reported by Robbins is a form of *Protoderma viride*.

As has been pointed out by Esmarch, this flora undoubtedly originated from the surface flora, but its persistence in the soil at such great depths is noteworthy. It is inconceivable that in undisturbed soil compact as clay, algae could be carried down very far by surface waters. There were no evidences of wormholes or penetration by surface organisms in these soils. This would seem to indicate that the algae are in a vegetative condition and actually grow in the soil.

The amount of growth in the various bottles can be taken to represent in a general way the abundance of the algae in the soil at the different depths, since the cultures were all kept under similar conditions. It was impossible to determine this from a microscopical examination of the soil samples, because the algae were present in such small quantities that they could not be easily found among the soil particles. The greatest growth was never at the surface but at a depth of 5–60 cm. This was due probably to the dry conditions existing at the surface. From 60 to 100 cm. the amount of algae in the cultures gradu-

ally became less. In some cases this was due to the disappearance of some of the algal forms but usually the amount of an individual form also decreased. *Protoderma* was always more abundant towards the surface than at the greater depths.

The time in which the growth was first perceptible in the cultures varied from about 3 weeks to 3 months. This was dependent no doubt upon the amount of algae and also upon the amount in a vegetative condition in the soil. Obviously, vegetative cells would produce a growth in less time than spores.

The resistance of these algae to desiccation was demonstrated in series B–F inclusive, in which the cultures were allowed to evaporate for a period of about 18 months, from March, 1917, to November, 1918. The cultures became quite dry within several months, so that the algae were exposed to desiccating conditions for about 12 months. In the fall of 1918, the cultures were reëxamined and in most cases the algae seemed in a healthy condition. After the cultures were moistened vigorous growth occurred again. Especially noticeable was the fact that while many of the vegetative cells of *Ulothrix* and *Stichococcus* were plasmolyzed, very few of the *Protoderma* cells showed any injurious effects.

The following is a list of the algae found in the cultures and the greatest depth at which they occurred:

Protoderma viride Kützing	100 cm.
Hantzschia amphioxys (Ehr.) Grun	100 cm.
Navicula atemoides Grun	100 cm.
Trochiscia?	80 cm.
Stichococcus bacillaris Nägeli	70 cm.
Oscillatoria amphibia Agardh	70 cm.
Cladophora sp	60 cm.
Ulothrix variabilis Kützing	60 cm.
Anabaena sp	20 cm.
Nitzschia Kützingiana Hilse	20 cm.
Nostoc muscorum Agardh	20 cm.
Oscillatoria chlorina Kützing	20 cm.
Oscillatoria subtilissima Kützing	20 cm.
Scytonema Hofmanni Agardh	20 cm.
Oscillatoria anoema (Kützing) Gomont	Surface
Oscillatoria formosa Bory	Surface
Oscillatoria splendida Greville	Surface

Artificial Subterranean Cultures

Very little work has been done on the effect of subterranean conditions upon the growth of algae. In studying the effect of light, culture media have been used which did not duplicate soil conditions. Esmarch determined the effect of light upon the growth of certain *Cyanophyceae* which he found in subterranean cultures. As shown above, these algae were grown on soil in the dark and examined from time to time. From his results, he concluded that these forms could live in darkness for a short period of time but after several weeks, in most cases, the cells showed effects due to the absence of light. The extent of this effect depended upon the nature of the soil and individual characteristics of the alga. Eventually only spores and heterocysts remained.

Since *Protoderma viride* was found to be universally present in the cultures, an attempt was made to determine the effect of subterranean conditions upon its growth. The foregoing results showed that it could exist in undisturbed soils for a depth of one meter at least, thus being capable of living for long periods of time in the absence of light. In order to determine the effect of these conditions upon its growth, the following experiments were performed:

One culture was set up January 15, 1919. A small amount of sterile clay was put into a sterile glass cylinder about 2 cm. in diameter and 1 m. long. The soil was moistened with sterile water and a piece of sterile filter-paper that had been inoculated with *Protoderma* derived from culture H9′ was placed on top. Alternate layers of soil, moistened with water, and inoculated filter-paper were added until the tube was filled. A sterile cotton plug was used to cork the top, so that some aëration could take place. The tube was then sunk in the ground to the depth of 1 m., thus allowing the culture to grow under somewhat natural conditions. A flower-pot was placed over the top to protect the cotton plug.

Other cultures were set up on February 1, 1919. The actual aëration conditions occurring in the soil were more nearly duplicated in these than in the previous culture, since small sterile

cheese-cloth bags were filled with sterile clay soil which had been inoculated with *Protoderma* from culture H8′. These were placed in 6 sterile atmometer tubes which were then filled with sterile soil and enough sterile water to moisten. The tubes were corked and put in the ground at a depth of about 25 cm.

The tube in the first experiment was examined after about five months, on June 20, 1919, and *Protoderma* was found upon the filter-paper and also to some extent in the soil. Abundant growth was obtained in the part of the tube at the surface where some light had entered because the flower-pot did not at all times fit closely over the top. The rate of growth beneath the surface was much less, but even at a depth of 1 m. the algal cells were brilliantly green and healthy and had grown to some extent into the soil. This is comparable to the growth occurring under natural conditions, because in the soil samples taken algal growth was so scant that it could not be detected with the naked eye. In all cases the cells were either in plates or small and large single cells, some of which resembled *Protococcus*.

In the second experiment, the tubes were examined after different intervals of time. This method proved to be less satisfactory than the above due to the difficulty of finding the scattered algal cells among the soil particles. Thus, negative results would not necessarily mean that the algal cells had disintegrated.

Culture No.	Time examined	Interval	Result
1	Feb. 12, 1919	11 days	Some brown and colorless cells.
2	Feb. 18, 1919	17 days	No cells.
3	Feb. 24, 1919	23 days	Numerous green cells.
4	June 20, 1919	140 days	No cells.
5 and 6	June 27, 1919	147 days	Numerous green cells (2 tubes examined).

The above results agree with those in the first experiment. The fact that numerous *Protoderma* cells were found in the cul-

ture kept underground for the longest period of time would seem to indicate that the first culture examined was not a normal one. In cultures 2 and 4, the cells may have been overlooked, owing to the difficulty of finding them among the soil particles.

Since there is now in progress a detailed physiological study of *Protoderma* which will attempt to determine its possible function in the soil together with the influence of various environmental factors on its life history and growth, no reference to the literature nor further discussion of the problem need be given here. It is hoped that a subsequent paper on the subject may be published in the Annals within a short time.

CULTURE EXPERIMENTS WITH MELAMPSORA IN JAPAN

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Culture experiments with the heteroecious rust *Melampsora* have been extensively undertaken by investigators in different countries, especially in Germany. However, owing to the diversity of local conditions and also the variability of the fungi themselves, the data which are secured in one country can not readily be accepted in others. The life histories of such heteroecious forms, therefore, require to be worked out for each country separately.

In the writer's first article ('15) of this study, the inter-relationships between the different spore types of a few species of *Melampsora* and the host species of *Salix* plants were for the first time reported in Japan. The life histories which have already been determined and reported by me are for the five species arranged in the following key:

Key to the Species

A. Teleutospores subepidermal.
  a. Teleutospores amphigenous and uredospores hypophyllous 1
    Uredospores 15–20×12–15 μ. Teleutospores 26–37×8–13 μ.
    On *Salix opaca* Anders ..... *1. M. Larici-opaca* Miyabe and Matsumoto
  b. Teleutospores and uredospores mostly hypophyllous 2
    Uredospores 15–22×12–16 μ. Teleutospores 18–40×7–11 μ.
    On *Salix Miyabeana* v. Seem .....
    ..... *2. M. Larici-Miyabeana* Miyabe and Matsumoto

B. Teleutospores subepidermal, frequently subcuticular.
  a. Teleutospores amphigenous, but mostly epiphyllous. Uredospores hypophyllous 3, 4
    Uredospores 13–19×11–15 μ. Teleutospores 30–58×8–13 μ.
    On *Salix viminalis* L. ..... *3. M. Larici-epitea* Kleb.
    Uredospores 14–18×11–14 μ. Teleutospores 30–55×8–14 μ.
    On *Salix daphnoides* Vill. ..... *4. M. Larici-daphnoides* Kleb.

C. Teleutospores subcuticular.
  a. Teleutospores amphigenous, but mostly epiphyllous. Uredospores mostly hypophyllous, frequently amphigenous 5
    Uredospores 18–29×12–16 μ. Teleutospores 20–30×8–12 μ.
    On *Salix jessoensis* v. Seem ..... *5. M. yezoensis* Miyabe and Matsumoto

Since additional cultural results with *Melampsora* on species of *Salix* and *Populus* have been secured since the positive results in 1915, on the species mentioned above, supplementary notes are given later in the present paper.

MELAMPSORA ON SALIX URBANIANA V. SEEM.

In April, 1916, a large number of inoculations of *Larix decidua* with *Melampsora* obtained from *Salix Urbaniana* were undertaken for the purpose of verifying the results published in my earlier paper. Positive results were readily secured on *Larix decidua*, as shown in table I, while on the remaining species the inoculations were unsuccessful.

In May, 1916, several series of infection experiments were performed by the inoculation of *Salix Urbaniana* with the caeomaspores which had been produced on *Larix decidua*. After a week positive results were secured.

TABLE I
SHOWING THE RESULTS OF INOCULATION WITH MELAMPSORA FROM SALIX URBANIANA

Inoculation material	Species inoculated	Date of inoculation	Result	Date of first sori
Teleutospores from *Salix Urbaniana*	*Larix decidua*	April 28	+	May 15
Salix Urbaniana	*Salix Urbaniana*	April 28	−	....
Salix Urbaniana	*Allium Cepa*	April 28	−	....
Salix Urbaniana	*Chelidonium majus*	April 28	−	....
Caeomaspores from *Larix decidua*	*Salix Urbaniana*	May 18	+	May 25

From the experiments it is evidently established that the species on *Salix Urbaniana* found in Sapporo, Japan, is heteroecious and must have its aecidial stage on *Larix* sps. In consideration of the evidence given, as well as that which follows, I consider this a new species, and the accompanying diagnosis and notes are offered:

Melampsora Larici-Urbaniana Matsumoto, n. sp.

Aecidiospores. Caeomata hypophyllous, scattered, pale orange-yellow with yellow spots on the upper surface, roundish

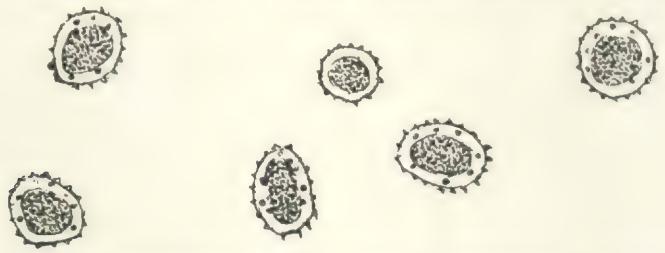

Fig. 1. *Melampsora Larici-Urbaniana.* Caeomaspores. Camera lucida drawing ×460.

or oblong; spores roundish or oval, finely echinulate, 15–26× 13–19 μ; membrane hyaline, 3–4 μ thick.

Uredospores. Sori hypophyllous, densely scattered over the whole lower surface, with yellow spots showing on the upper

Fig. 2. *Melampsora Larici-Urbaniana.* Uredospores. Camera lucida drawing ×460.

surface, seated on small orange-yellow spots; spores mostly oval, sometimes oblong or roundish, with a more or less elongated stalk, 15–26×12–17 μ; membrane hyaline, echinulate, without perceptible germ pore; paraphyses capitate, with a thin pedicel (3–4 μ), 50–70×18–22 μ.

Teleutospores. Sori hypophyllous, dark reddish brown, scattered over the whole surface or confluent in excessive crusts, covered by the epidermis; spores prismatic, rounded at both

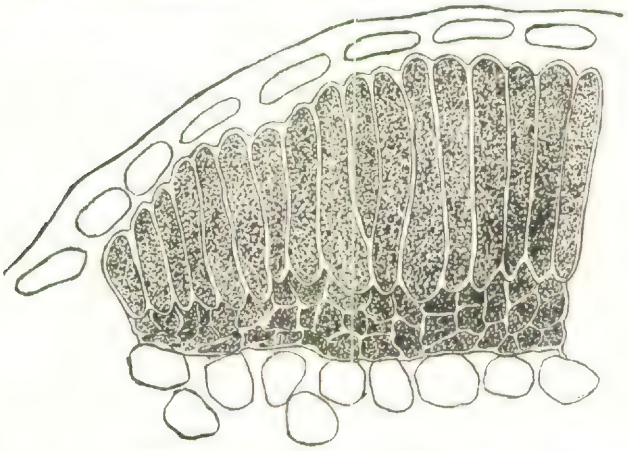

Fig. 3. *Melampsora Larici-Urbaniana.* Teleutospores. Camera lucida drawing ×460.

ends, 38–70×9–15 μ; membrane somewhat brown, uniformly thin, without an evident germ pore; contents orange-red; sporidia spherical, 9–15 μ.

Caeomata on *Larix decidua;* uredo- and teleutospores on *Salix Urbaniana.*

This species is more or less related to *Melampsora Laricipentandrae* Kleb., as shown by the position of the teleutospore layer, by the thickness of the apical cell-wall, and by having the *Caeoma* stage on the leaves of *Larix* sps. On the other hand, there are characteristic differences, as follows: (1) The uredospore layer of the fungus in question is hypophyllous, while that of the other is epiphyllous; (2) The uredospores of our species are considerably shorter than the spores described by Klebahn (26–44×12–16 μ); (3) The teleutospores of our form are so decidedly larger that even the smallest can hardly be compared with the largest of the spores described by Klebahn (28–38× 6–11 μ).

MELAMPSORA ON POPULUS BALSAMIFERA

As already stated in my previous paper, the author noticed an abundance of *Caeoma* on the leaves of *Chelidonium majus* at

Nakajima Park, Sapporo, where there were growing many species of *Populus* badly attacked by the *Melampsora* rusts. These indications, in the light of results obtained by Klebahn in Germany, induced me to assume that there might be some relationship between the *Caeoma* on *Chelidonium* and the *Melampsora* on some species of *Populus*. However, in the inoculation of *Chelidonium* with *Melampsora* from *Populus* (1915), no light could be thrown on this subject; therefore in the following year additional cultures were made, but these also failed to yield any positive results.

After these successive negative results, the writer made cultures with species of *Melampsora* from *Populus balsamifera* on *Larix leptolepis*, *Larix decidua*, *Ribes grossularia*, and *Allium Cepa*. A study of the data in table II shows that the sporidia of the rust on *Populus balsamifera* infect *Larix leptolepis* and *Larix decidua* without any apparent preference, while on the remaining plants they prove to be quite ineffective.

TABLE II

SHOWING THE RESULTS OF INOCULATIONS WITH MELAMPSORA FROM POPULUS BALSAMIFERA

Inoculation material	Species inoculated	Date of inoculation	Result	Date of first sori
Teleutospores from				
Populus balsamifera	*Larix leptolepis*	May 2	+	May 18
Populus balsamifera	*Larix decidua*	May 2	+	May 19
Populus balsamifera	*Ribes grossularia*	May 2	−	....
Populus balsamifera	*Allium Cepa*	May 2	−	....
Caeomaspores from				
Larix decidua	*Populus balsamifera*	May 24	+	June 8

The species can properly be regarded as *Melampsora Laricipopulina* Kleb. on account of the position of the uredo- and teleutospore layer and the relationship between the different spore forms and the host plants. The author observes some

difference in size between both the caeoma- and the teleutospores of species from the two sources, but these points alone are not sufficient to be considered as of specific importance.

The characterization of the species is as follows:

Melampsora Larici-populina Kleb.

Aecidiospores. Caeomata hypophyllous, single or in groups, with yellow spots on the upper surface, roundish or oblong, 1–1.5 mm. in diameter, orange-red, pulverous; spores roundish or oval, finely and densely verruculose, 22–37×18–27 μ.

Uredospores. Sori mostly hypophyllous, seated on yellow spots, scattered over the whole surface, orange-yellow, pulverous; spores oval or elongated, 26–40×16–22 μ; membrane hyaline, finely echinulate, without perceptible germ pore; paraphyses capitate, with a slender pedicel, 16–22×55–80 μ.

Teleutospores. Sori epiphyllous, frequently hypophyllous, dark reddish brown, scattered or in groups over the whole surface, covered by the epidermis; spores cylindrical or somewhat wedge-shaped, 18–48×8–12 μ; membrane clear brown, uniformly thin, without an evident germ pore; sporidia spherical.

Caeomata on *Larix leptolepis* and *Larix decidua;* uredo- and teleutospores on *Populus balsamifera.*

MELAMPSORA ON SALIX BABYLONICA

When negative results were obtained as to any relationship between *Caeoma* on *Chelidonium* and *Melampsora* on *Populus,* the author performed a new experiment by inoculating *Chelidonium majus* with teleutospore material obtained from several species of *Salix* and *Populus.*

As will be shown in the data of table III, successful results were only secured by sowing the teleutospore material obtained from *Salix babylonica.*

As may be easily seen, the species on *Salix babylonica* requires *Chelidonium majus* for complete development of its entire life cycle, but owing to the fact that no return infections to *Salix* sps. have been made, the subject has not yet been completely established.

The aecidial stage resulting from the successful inoculation

TABLE III

SHOWING THE RESULTS OF INOCULATIONS OF CHELIDONIUM WITH TELEUTOSPORES

Inoculation material	Date of inoculation	Result	Date of first sori
Teleutospores from			
Salix Capraea	June 10	−	....
Salix babylonica	June 10	+	June 21
Populus nigra	June 10	−	....
Populus balsamifera	June 10	−	....

with the teleutospores obtained from *Salix babylonica* may be described as follows:

Aecidiospores. Caeomata hypophyllous, clustered or isolated, with yellow spots on the upper surface, small, roundish; spores roundish or oblong, 14–18×13–17 μ; membrane hyaline, finely verruculose.

Melampsora on Salix Capraea

From the fact that our Japanese *Melampsora* on *Salix Capraea* is morphologically more or less similar to *Melampsora Larici-Capraearum* described by Klebahn, the writer was inclined to assume that the first-named form might have its aecidial stage on *Larix* sps., consistent with the observations made in Germany.

In April, 1916, a large number of experiments were undertaken by inoculating *Larix decidua* with sporidia obtained from the teleutospore stage on *Salix Capraea*, but no successful result has been secured. According to von Tubeuf, successful results were obtained by sowing *Caeoma Abietis pectinatae* upon *Salix Capraea*. I have been unable to establish this relationship.

Summary

1. A *Melampsora* on *Salix Urbaniana* requires *Larix* sps. for the completion of its life cycle. For this species the name *Melampsora Larici-Urbaniana* Matsumoto is proposed.

2. A *Melampsora* on *Populus balsamifera* found in Japan is identified with *Melampsora Larici-populina* described by Klebahn in Germany.

3. A *Melampsora* on *Salix babylonica* has its *Caeoma* stage on the leaves of *Chelidonium majus*. Owing to the lack of infection experiments with the alternate host the relationship has not yet been completely established.

4. A *Melampsora* on *Salix Capraea* seems to have a *Caeoma* stage neither on the leaves of *Larix* sps. nor *Abies* sps.

The author wishes to express here his heartiest thanks to Dr. K. Miyabe to whom he is indebted for many valuable suggestions, likewise to Dr. B. M. Duggar for his kindly advice and criticism. Thanks are also due Dr. G. T. Moore for the privileges of the library.

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GENERAL INDEX TO VOLUME VI

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italic;* and previously published scientific names and all other matter, in ordinary type.

A

Aleurodiscus tabacinus, 261
Algal flora, A subterranean, 281
Allen, E. R.: Some conditions affecting the growth and activities of Azotobacter chroococcum, 1; and Davisson, B. S. An all-glass nitrogen apparatus, 45
Allium Cepa, 310
Amidase in fungi, 197
Amino-nitrogen, determination of, 75
Ammonia, determination of, 75
Anabaena sp., 302
Apparatus: nitrogen-distilling, *24;* an all-glass nitrogen, 45, *48*
Arcangeliella, 49; Borziana, 49, 53; **caudata**, 49, 51; **Soderstromii**, 52
Arcangeliella, Gymnomyces, and Macowanites in North America, 49
Armillaria mellea, enzyme action in, 193
Aspergillus glaucus, growth of, in mixed cultures, 183, *192*
Aspergillus niger: germination and hydrogen ion concentration of, 207; growth of, in mixed cultures, 183, *192*
Atkinsonii (Lachnocladium), 277
Auximones, 6
Azotobacter chroococcum: in relation to wood decay induced by fungi, 116; Some conditions affecting the growth and activities of, 1, *44*

B

Bacillus radicicola, 1
Bacillus species in relation to wood decay induced by fungi, 116
Bacteria: nitrifying, 1; nitrogen-fixing, 1; The relation of, to cellulose fermentation induced by fungi, with special reference to the decay of wood, 93
Bacterium mycoides in relation to wood decay induced by fungi, 116
Berkeleyi (Veluticeps), 260
bicolor (Lachnocladium), 274
bicolor (Clavaria), 274
bicolor (*Clavaria*), 274
Biological fluids, pigmented, and H ion determination, 61, 179
Bonia, 262
Bonia, 261; *flava*, 262
Botrytis cinerea, germination and hydrogen ion concentration of, 207
brasiliense (Lachnocladium), 268, *280*
brasiliensis (Protomerulius), 175
brunneoleuca (Mycobonia), 263
brunneoleucum (*Hydnum*), 263
Burt, E. A. An edible garden Hebeloma, 171; Merulius in North America, supplementary notes, 143; Protomerulius Farlowii Burt, n. sp., 175; The Thelephoraceae of North America, XI, 253

C

Calcium hypochlorite solution, treatment of seeds with, 151
Carbohydrases in fungi, 194
Carboxyl groups: determination of, 76; fate of, 84
cartilagineum (Lachnocladium), 269, *280*
Cellulose fermentation, relation of bacteria to, 93
cervina (*Clavaria*), 273
cervinum (Lachnocladium), 273, *280*
Chelidonium majus, 312
Cladophora sp., 286
Clavaria, 267; bicolor, 274; *bicolor*, 274; *cervina*, 273; *fragrans*, 270; *furcellata*, 274; *pallida*, 273; *Peckii*, 274; *vestipes*, 274
Colletotrichum lindemuthianum, germination and hydrogen ion concentration of, 207
Colloids, protective, in nutrient media, 35
Colorimeter, The use of the, in the indicator method of H ion determination with biological fluids, 61
Coniophora cerebella: growth of, in mixed cultures, 183; growth of, on liquid media, 138
Corticium incarnatum pinicolum, 256; roseolum, 256; *tabacinum*, 261
Culture experiments with Melampsora in Japan, 309
Cultures: mixed, 183, *192;* subterranean, methods of obtaining, 284, algae found in, 304, artificial, 305

D

Daedalea confragosa: enzyme action in, 193; growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Daedalea quercina: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Davis, Anne W., Duggar, B. M., and. Seed disinfection for pure culture work: the use of hypochlorites, 159
Davisson, B. S., Allen, E. R. and. An all-glass nitrogen apparatus, 45
Deamination in the tyrosinase reaction, 80
Diastase activity in relation to stage of development and carbohydrate content of the tuber of Solanum tuberosum, 223
Disinfection, seed, 147, 159
Dodge, C. W.: Tyrosin in the fungi: chemistry and methods of studying the tyrosinase reaction, 77; Duggar, B. M., and. The use of the colorimeter in the indicator method of H ion determination with biological fluids, 61; Zeller, S. M., and. Arcangeliella, Gymnomyces, and Macowanites in North America, 49
Dubosq colorimeter, 179
Duggar, B. M.: The micro-colorimeter in the indicator method of hydrogen ion determination, 179; and Davis, Anne W. Seed disinfection for pure culture work: the use of hypochlorites, 159; and Dodge, C. W. The use of the colorimeter in the indicator method of H ion determination with biological fluids, 61; Zeller, S. M., Schmitz, H., and. Studies in the physiology of the fungi. VII, Growth of wood-destroying fungi on liquid media, 137
Dussii (Epithele), 265
Dussii (*Hypochnus*), 265
Dussii (*Peniophora*), 265

E

Eichleriana (Tulasnella), 255
Emulsin in fungi, 196
Enzyme action: and tuber development, 223; in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr., 193
Epithele, 264; Dussii, 265; **sulphurea,** 265
Epithele, 264
erectum (Lachnocladium), 276, *280*
Eriocladus, 266
Esterases in fungi, 194

F

Farlowii (Protomerulius), 176
flava (*Bonia*), 262
flava (Mycobonia), 262
flava (*Peziza*), 262
flavum (*Grandinioides*), 262, 264
flavum (*Hydnum*), 262
Fomes pinicola in relation to decay of wood, 112
Formaldehyde, treatment of seeds with, 153
fragrans (*Clavaria*), 270
Fungi: enzyme action in certain, 193; germination of spores of, in relation to H ion concentration, 201; mixed cultures of, 183; relation of bacteria to cellulose fermentation induced by, 93; Studies in the physiology of the, VI, 93, VII, 137, VIII, 183, IX, 193, X, 201; Tyrosin in the, 71; wood-destroying, on liquid media, 137
furcellata (*Clavaria*), 274
furcellatum (Lachnocladium), 274, *280*
Fusarium sp., germination and hydrogen ion concentration of, 207
fusco-violacea (Tulasnella), 258

G

Germination of the spores of certain fungi in relation to hydrogen ion concentration, 201
Grandinioides, 262; *flavum,* 262, 264
guadalupensis (*Pterula*), 277
guadelupense (Lachnocladium), 277
guadelupense (*Merisma*), 277
Gymnomyces, 54; **Gardneri,** 54; pallidus, 54, 55; seminudus, 54, 56

H

Hantzschia amphioxys, 296
Hebeloma, an edible garden, 171
Hebeloma **hortense,** 172, *174*
hortense (Hebeloma), 172, *174*
Hydnangium Soderstromii, 52
Hydnum brunneoleucum, 263; *flavum,* 262
Hydrogen: electrode, 61; ion concentration, germination of the spores of certain fungi in relation to, 201, relation between, and growth of fungi in mixed cultures, 188; ion determination, the use of the colorimeter in the indicator method, with biological fluids, 61, micro-colorimeter in the indicator method of, 179
Hymenochaete veluticeps, 260
Hypochlorites in seed disinfection, 159
Hypochnus Dussii, 265; *violeus,* 256

I

incarnata (Pachysterigmata), 256
incarnata (*Tulasnella*), 256
incarnatum pinicolum (*Corticium*), 256
Indicator method of H ion determination, 61, 179
Isaria sp., growth of, in mixed cultures, 183, *192*

K

Karrer, J. L., Moore, G. T., and. A subterranean algal flora, 281
Kober colorimeter, 66, 181

L

Lachnocladium, 266; Atkinsonii, 277, **bicolor,** 274, *280;* brasiliense, 268, *280;* cartilagineum, 269, *280;* cervinum, 273, *280;* **erectum,** 276, *280;* furcellatum, 274, *280;* guadelupense, 277; Micheneri, 270, *280;* odoratum, 278; *odoratum,* 270; *pallidum,* 273; semivestitum, 271, *280;* subsimile, 272, *280*
Larix decidua, 310; leptolepis, 313
Lentinus lepideus: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Lenzites saepiaria, germination and hydrogen ion concentration of, 207
Lenzites vialis: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138; in relation to decay of wood, 112

M

McGinty, R. A. Diastase activity in relation to stage of development and carbohydrate content of the tuber of Solanum tuberosum, 223
Macowania, 56
Macowania, 56; *agaricina,* 58
Macowanites, 56; agaricinus, 56, 58; **echinosporus,** 57
Matsumoto, T. Culture experiments with Melampsora in Japan, 309
Media, liquid, growth of wood-destroying fungi on, 137
Melampsora, 309; Larici-daphnoides, 309; Larici-epitea, 309; Larici-Miyabeana, 309; Larici-opaca, 309; Larici-pentandrae, 312; Larici-populina, 314; **Larici-Urbaniana,** 311; yezoensis, 309
Mercuric chloride, treatment of seeds with, 156
Merisma, 267
Merisma quadelupense, 277
Merulius hirsutus, 145; incarnatus, 143; lacrymans, 145, growth of, in mixed cultures, 183, *192,* growth of, on liquid media, 138; pinastri, growth of, in mixed cultures, 183, *192,* growth of, in liquid media, 138; rimosus, 145; rubellus, 143, 145
Micheneri (Lachnocladium), 270, *280*
Micro-colorimeter, The, in the indicator method of hydrogen ion determination, 179
Moore, G. T. and Karrer, J. L. A subterranean algal flora, 281
Mycobonia, 253, 261; brunneoleuca, 263; flava, 262

N

Navicula atemoides, 296
Nitrogen: apparatus, 24, An all-glass, 45, *48;* fixation by Azotobacter chroococcum, 15; methods, 23
Nitzschia Kützingiana, 296
Nostoc muscorum, 296

O

odoratum (Lachnocladium), 278
odoratum (*Lachnocladium*), 270
Oscillatoria amphibia, 296; anoema, 296; chlorina, 296; formosa, 296; splendida, 296; subtilissima, 296

P

Pachysterigma, 253
Pachysterigmata incarnata, 256
pallida (*Clavaria*), 273
pallidum (*Lachnocladium*), 273
Peckii (*Clavaria*), 274
Penicillium cyclopium, germination and hydrogen ion concentration of, 207
Peniophora Dussii, 265
Peziza flava, 262
Phenol groups: determination of, 76; fate of, 84
Pleurotus sapidus: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Polyporus lucidus: enzyme action in, 193; growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Polystictus hirsutus: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Polystictus versicolor: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138; in relation to decay of wood, 112
Populus balsamifera, 312
Proteases in fungi, 198
Protoderma viride, 286
Protomerulius brasiliensis, 175; **Farlowii,** 175, 177

Prototremella, 253; *Tulasnei*, 256
Pterula, 267; setosa, 278; *guadalupensis*, 277

R

Ribes grossularia, 313
roseolum (Corticium), 256

S

Salix babylonica, 314; Capraea, 315; daphnoides, 309; jessoensis, 309; Miyabeana, 309; opaca, 309; Urbaniana, 310; viminalis, 309
Schmitz, H.: Studies in the physiology of the fungi. VI, The relation of bacteria to cellulose fermentation induced by fungi, with special reference to the decay of wood, 93; Zeller, S. M., and. Studies in the physiology of the fungi. VIII, Mixed cultures, 183; and S. M. Zeller. Studies in the physiology of the fungi. IX, Enzyme action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr., 193; Zeller, S. M., Duggar, B. M., and. Studies in the physiology of the fungi. VII, Growth of wood-destroying fungi on liquid media, 137
Scytonema Hofmanni, 296
Seed disinfection for pure culture work, 147, 159
semivestitum (Lachnocladium), 271, *280*
setosa (Pterula), 278
Solanum tuberosum, diastase activity in relation to stage of development and carbohydrate content of the tuber of, 223
Spore germination and H ion concentration, 201
Standard solutions, 62
Stichococcus bacillaris, 286
Studies in the physiology of the fungi. VI, The relation of bacteria to cellulose fermentation induced by fungi with special reference to the decay of wood, 93; VII, Growth of wood-destroying fungi on liquid media, 137; VIII, Mixed cultures, 183; IX, Enzyme action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr., 193; X, Germination of the spores of certain fungi in relation to hydrogen ion concentration, 201
subsimile (Lachnocladium), 272, *280*
Sugar determination, 21, 241
sulphurea (Epithele), 265

T

tabacina (Veluticeps), 261
tabacinum (*Corticium*), 261
tabacinus (*Aleurodiscus*), 261
Tannase in fungi, 196
Thelephoraceae of North America, The, XI, 253
Trametes Peckii: growth of, in mixed cultures, 183, *192;* growth of, on liquid media, 138
Trochiscia, 286
Tulasnei (*Prototremella*), 256
Tulasnei (*Tulasnella*), 256
Tulasnella, 253; Eichleriana, 255; fuscoviolacea, 258; *incarnata*, 256; *Tulasnei*, 256; violea, 256
Tyrosin in the fungi: chemistry and methods of studying the tyrosinase reaction, 71
Tyrosinase reaction, 71

U

Ulothrix variabilis, 286
Urease in fungi, 197

V

Veluticeps, 253, 259; Berkeleyi, 261; tabacina, 261
Veluticeps, 259
veluticeps (*Hymenochaete*), 260
vestipes (*Clavaria*), 274
violea (Tulasnella), 256
violeus (*Hypochnus*), 256

W

Webb, R. W. Studies in the physiology of the fungi. X, Germination of the spores of certain fungi in relation to hydrogen ion concentration, 201
Wood: decay induced by fungi, 93; effects of autoclaving, 94
Wood-destroying fungi: enzyme action in, 195; on liquid media, 137

Y

Young, H. C. Seed disinfection for pure culture work, 147

Z

Zeller, S. M.: and Dodge, C. W. Arcangeliella, Gymnomyces, and Macowanites in North America, 49; and Schmitz, H. Studies in the physiology of the fungi. VIII, Mixed cultures, 183; Schmitz, H., and. Studies in the physiology of the fungi. IX, Enzyme action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr., 193; Schmitz, H., and Duggar, B. M. Studies in the physiology of the fungi. VII, Growth of wood-destroying fungi on liquid media, 137

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Missouri Botanical Garden

Contents
February, 1919

Some Conditions Affecting the Growth and Activities of Azotobacter chroococcum ..... E. R. Allen 1-44

An All-Glass Nitrogen Apparatus ..... E. R. Allen and B. S. Davisson 45-48

Arcangeliella, Gymnomyces, and Macowanites in North America ..... S. M. Zeller and C. W. Dodge 49-59

The Use of the Colorimeter in the Indicator Method of H Ion Determination with Biological Fluids ..... B. M. Duggar and C. W. Dodge 61-70

Tyrosin in the Fungi: Chemistry and Methods of Studying the Tyrosinase Reaction ..... C. W. Dodge 71-92

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